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A Rapid Estimation of Prednisolone Produced by Fermentative Oxidation

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Summary. An analytical method has been devised for the rapid determination of prednisolone produced in a fermentative oxidation process. It allows frequent checking of concentrations of the prednisolone formed during the oxidation of hydrocortisone.

The estimation of prednisolone in the presence of hydrocortisone is based upon the diversity of colour shown by the chromogens produced from these steroids by sulphuric acid and ethanol. Using the optimal reagent mixture and conditions, the interference of hydrocortisone is negligible. The steroids are extracted from the fermentation broth with ethyl acetate. One test takes about 20 min.

Introduction

In microbiological transformations of steroids, the proper selection of a suitable organism, favourable fermentation conditions, and efficient methods of recovery necessarily require, and are supplemented by, an adequate analytical check if the yields of the steroid products are to be kept on a consistently high level. Because fermentative oxidations do not take long to go to completion (5–10 h), and continuation of oxidation then means loss of substantial quantities of the product, it became necessary to look for some analytical procedure which allowed frequent tests of the broth within the time of one fermentation run.

Many methods are mentioned in the literature as suitable for the qualitative and quantitative determination of steroids produced in the course of microbiological transformations. As a rule, these methods include a chromatographic separation. A general survey of these is given by Neher.[1] Methods based upon chromatographic separation furnish adequate information about the composition of mixtures of steroid compounds. They cannot, however, quantitatively register the transformations when these

are still in progress and give, owing to the time consumed by chromatography, only an outdated picture of them.

In our laboratories research has been undertaken to find circumstances under which it becomes possible to determine the quantity of a chosen steroid in the presence of other steroids and the ingredients of the fermentation liquor, without having to resort to chromatography, thus making it possible to keep track of the progress of fermentative oxidations. Recent publications from this laboratory[2, 3] deal with such methods of estimation for the microbiological production of $\Delta^{1,4}$-androstadiene-3,17-dione and 17-α-oxi-11-desoxicorticosterone. Here an analytical control method used in the manufacture of prednisolone from hydrocortisone by fermentative oxidation, i.e. 1-dehydrogenation, will be described.

Several micro-organisms and methods are known[4] for the 1-dehydrogenation of steroids, and among these the methods of Shull[5] and Nobile[6] may be mentioned as having served as models in our researches.

Experimental

The task was to determine 40- to 1,000-μg quantities of prednisolone in mixtures with hydrocortisone present in a prednisolone : hydrocortisone ratio of 1:10. Reactions likely to lead to chromogen formation were considered, and conditions which led to sufficiently different absorption spectra for the differentiation of the steroids in question were worked out.

The formation of chromogens of adrenal steroids and related compounds with concentrated sulphuric acid has been described by Zaffaroni.[7, 8] According to Linford,[9] a dilution of the yellow chromogen with methanol brings about a shift of the absorption towards the red end of the spectrum where there is less interference from the background.

Fig. 1. shows absorption curves plotted for hydrocortisone and prednisolone when their chromogens had been formed with concentrated sulphuric acid. The divergence of these curves is not sufficient to allow their use in an analysis of prednisolone, nor could this situation substantially be improved upon by the subsequent dilution of the chromogens with methanol.

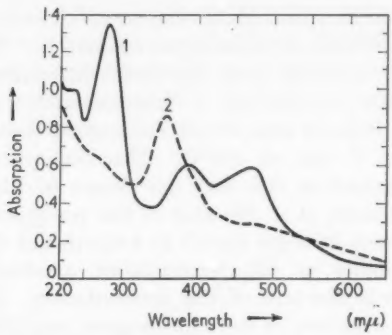

Fig. 1. Absorption spectra of the chromogens (by the Zaffaroni[8] method) of hydrocortisone (1) and prednisolone (2). Extinctions measured in a 10-mm cuvette. Concentration of steroid = 200 μg in 8 ml of the concentrated sulphuric acid

In further experiments absorption was investigated in relation to chromogen formation with sulphuric acid reagents made by the addition of varying amounts of water, methanol, or ethanol; and in relation to temperature and duration of the chromogen-forming reactions. These experiments have shown that considerable influence can be exerted upon the absorption characteristics of the

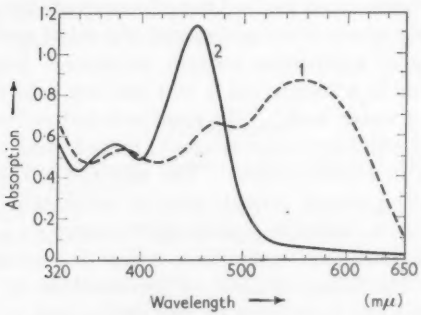

Fig. 2. Absorption spectra of the chromogens (by the ethanol–sulphuric acid method) of prednisolone (1) and hydrocortisone (2). Extinctions measured in a 10-mm cuvette. Concentration of steroid = 400 μg in 8 ml of the reaction mixture

chromogens of the two steroids by the composition of the reagent and by the conditions of chromogen formation. Evaluation of spectra has shown that the most significant difference between the absorptions of the two steroids is obtained with a reagent composed of 55 vol. ethanol and 45 vol. concentrated sulphuric acid, in a reaction of 5 min at 100°C. The absorption spectra of chromogens obtained in this way are shown in Fig. 2. At the absorption maximum (λ = 560 mμ) of the prednisolone chromogen, hydrocortisone does not absorb to a significant degree, and its influence diminishes as the fermentation proceeds, becoming negligible towards the end of the fermentation. Five minutes suffice for the formation of the chromogens, and this contributes to the rapidity of the test.

For the separation of the steroids from the fermentation broth, one shaking-out with 2 vol. of ethyl acetate extracted 96 per cent of its steroid content. After drying, an aliquot of this ethyl acetate solution is evaporated to dryness and the residue is taken up in the chromogenic reagent.

Procedure

The fermentation liquor originally contains 200 μg/ml hydrocortisone. For one test a 10·0-ml portion of the broth is transferred to a 100-ml separating funnel and 20·0 ml of analytical grade ethyl acetate is added. After vigorous shaking and standing, the aqueous phase is discarded and the ethyl acetate phase is dried with 2 g of anhydrous sodium sulphate. Four millilitres of the clean fluid in a 20-mm o.d. × 100 mm test tube is evaporated to dryness on a water bath. The residue is redissolved in 8·00 ml of the ethanol–sulphuric acid reagent, mixed, and immersed for exactly 5 min in boiling water. The mixture is then cooled for 3 min in running water, mixed, and its extinction measured at λ = 560 mμ on a suitable spectrophotometer, e.g. a Unicam S.P.500. The measured extinction value indicates on a calibration chart the concentration of prednisolone in the sample. The reagent for the formation of the chromogen is prepared by adding 45·0 ml of 98 per cent sulphuric acid to 55·0 ml of ice-cold ethanol in small portions with constant stirring. This reagent is stable for about 2 weeks in a refrigerator at 0°C.

For calibration, 100 μg/ml stock solutions of both prednisolone and hydrocortisone in ethyl acetate are prepared. Mixtures of these in different ratios serve for the establishment of individual points on the reference chart. From the stock solutions, and their various mixtures, 20·0-ml portions are brought into equili-

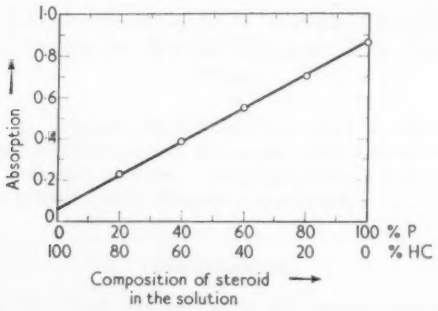

Fig. 3. Relationship between the absorption and the composition of steroid (prednisolone, P; hydrocortisone, HC) by the 'extraction and ethanol–sulphuric acid colour formed' method. Extinctions measured at $\lambda = 560$ mμ in a 10-mm cuvette

brium with 10·0 ml of water. The drying of the ethyl acetate phase, evaporation, and formation of the chromogens, are then carried out as before. Fig. 3 shows the correlation between absorption values at $\lambda = 560$ mμ and the steroid composition of the sample.

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An Improved pH Electrode Assembly for Pilot Plant and Plant Fermentors*

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Summary. A modified pH electrode assembly is described which is capable of steam sterilization and which can be installed in steel fermentors of all sizes in a variety of ways. Its design is such that electrode life is prolonged and contamination hazards are reduced.

Introduction

Reviewing the past dozen years, the main deterrent to continuous pH measurement and control in submerged pure culture fermentations has been the unavailability of electrode assemblies which are capable of withstanding steam sterilization. The problem has been circumvented in small fermentors by sterilizing the electrodes separately with an agent such as formaldehyde, ethylene oxide, or ultraviolet light.[1, 4, 5, 7] Use of one of these agents, however, involves introducing one or both of the sterile electrodes into the fermentor without contaminating the system. Obviously, this approach is not a desirable one for small-scale equipment and is completely impractical for plant-scale fermentors.

Although failure of both glass and reference electrodes has been observed when they are sterilized with steam under pressure, the more serious problems lie with the glass electrode. Two main types of failure have been noted when glass electrodes have been exposed to sterilization conditions, viz. failure due to breakage of the glass and failure due to moisture entering the electrode at the point of connection with the lead wire. The former problem has

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been resolved in recent years by the use of heat-resistant glass. The latter problem has, unfortunately, not been resolved completely as both Nelson *et al.*[2] and Denison *et al.*[6] have noted.

With the reference electrode the main cause of failure has undoubtedly been mechanical breakage. Of particular concern to all in the fermentation field, however, is the possibility of a contaminant entering the system from the electrolyte solution used in the salt bridge. This problem appears to have been resolved for all practical purposes by the addition of a suitable bactericidal agent such as phenol.

Two types of electrode assembly which can be steam-sterilized have been reported. The first of these, the immersion type, described by Nelson *et al.*[2] and Huang[8], has the advantages that sterilization of the entire system is ensured and that the pressure on the electrolyte reservoir is always equal to the fermentor pressure.

Studies made with an immersion type of assembly in our laboratory demonstrated that the design was not a practical solution to our needs because of the short life of the glass electrode as a result of failures associated with both steam-sterilization and mechanical breakage. Furthermore, the lack of versatility of the immersion assembly was a major disadvantage. The assembly was too large for convenient installation in 10-gal fermentors and too frequently broken in plant-scale equipment because of the turbulence and vibration encountered.

The second system (and in our opinion the more practical of the two) is that described by Denison *et al.*[6] He and his co-workers installed a standard pH flow assembly in an external leg on a 50-gal fermentor. They reported that the assembly was capable of withstanding steam-sterilization and was successfully used for both measurement and control of pH. The disadvantages of this system, including problems with contamination and mechanical breakage as well as lack of versatility of installation, are all associated with the basic design of the electrode glands.

In this paper a modified pH electrode assembly is described which is capable of steam-sterilization and which can be installed in steel fermentors of all sizes in a variety of ways. Its design is such that electrode life is prolonged and contamination hazards are reduced.

Design of Assembly

When experimentation with pH electrode systems was started some years ago it was felt that the most reasonable approach involved the adaptation of commercially available equipment to fermentation use. Studies with an immersion type of assembly revealed its basic disadvantages and attention was then focused on the electrode glands manufactured by Beckman Instruments, Inc., illustrated in Fig. 1. The problem of ensuring a positive flow of

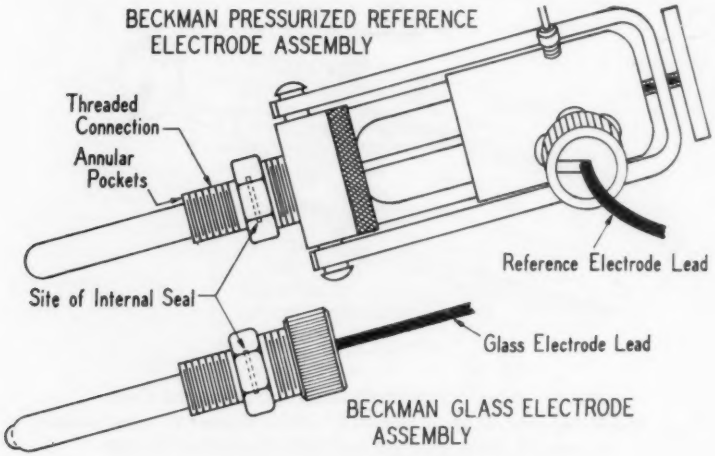

Fig. 1. Beckman pH electrode glands

electrolyte solution from the reference electrode against the pressure encountered in a fermentor was solved by the manufacturer by the application of external pressure to the electrolyte solution. No such provision was necessary for the glass electrode since the pressure problem does not exist. It was believed that the Beckman glands, if properly installed, would ensure longer life of both the glass and the reference electrodes since only a portion of the electrodes would need to be exposed to sterilization temperatures.

On the other hand, there are two basic disadvantages to the Beckman design. First, the annular pockets around the electrodes and the threaded connection tend to collect fermentation material and thus are potential sources of contamination. Secondly, the excessive protrusion of the electrodes through the gland assembly makes them susceptible to mechanical breakage. As a consequence, the glands were modified as illustrated in Fig. 2 to overcome these disadvantages.

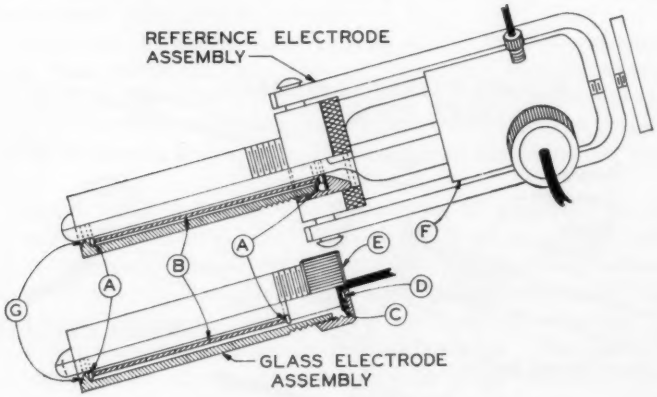

Fig. 2. Squibb modified glands (quarter-sectional view)
A, 'Quad' ring seals; B, gland sleeve; C, rubber washer; D, metal washer; E, cap; F, dome pressure assembly; G, chamfered edge

The basic differences between the modified and the original Beckman glands are seen when the two types are compared. The gland was lengthened so that only $\frac{1}{4}$ in.–$\frac{1}{2}$ in. instead of $2\frac{1}{2}$ in. of the sensitive tip of the electrode was in contact with the broth. The gland housing was modified so that it could be welded in place, eliminating the threaded connection. Additional 'quad' rings were inserted so that both assemblies were sealed at two points. In the glass electrode assembly one 'quad' ring was located at the pH-sensitive end and another was located at the top, below the plastic cap. The former compensated for variability in

electrode dimensions, and the latter contained the broth should the electrode tip be shattered. Two 'quad' rings were also placed in the reference electrode for the same reasons, the upper one adjacent to the gland sleeve and the lower one near the tip of the electrode. The electrodes used for the modified gland assemblies were standard Beckman industrial electrodes (manufacturer's code 19101 and 19700).

The relationship the various parts of the gland assemblies bear to each other is demonstrated in Fig. 2. Note that the electrode

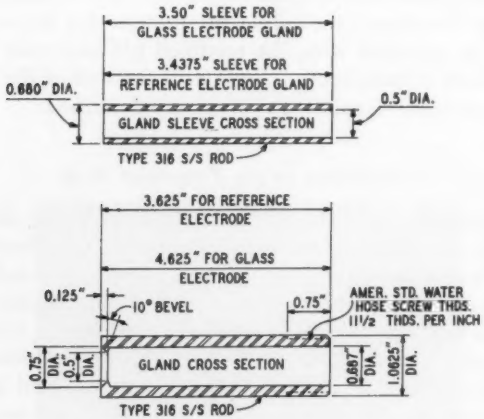

Fig. 3. Details of the Squibb glands and gland sleeves

is inside the gland sleeve and the gland sleeve inside the main body of the gland. The positions of the 'quad' rings, rubber washer, metal washer, cap, and dome assembly, are all shown. Note also that the inside edge of the gland next to the tip of the electrode is chamfered. The dimensions of the gland and the gland sleeve differ depending on which electrode they will be protecting. These details are given in Fig. 3. The inside diameter of the gland is set at 0·687 in. to permit use of a standard reamer to correct the distortion caused by welding during installation.

Method of Installation and Operation

The glands described are extremely versatile. They can be installed either directly in the side wall of a steel fermentor of any size or in an external circulating system. Although the operational advantages of the external system were recognized at the time these studies were initiated, it was felt that in small equipment the complications introduced by so equipping fermentors were not justified by the operating advantages gained. Consequently, developmental studies at the pilot and plant scale were concerned at the beginning with installation directly in the fermentor wall (direct-entry). Subsequently, an external circulating system was developed for use in production-size fermentors and this too was provided with the modified pH electrode assembly. Both methods of installation, directly in the wall of the fermentor and in an external circulating system, are described.

Installation in the Fermentor Wall

Our experience with the use of the glands in the direct-entry method of installation has been associated with fermentors of 10-gal, 100-gal, 200-gal, 1,300-gal and 17,000-gal total capacity. In all sizes of equipment two glands, one to hold the glass electrode and one to hold the reference electrode, are welded into the wall of the vessel as indicated in Fig. 4. Since fermentations are normally conducted at constant temperature, a third gland for a thermocompensator is unnecessary. The glands are installed at a minimum angle of 15° with the horizontal to ensure that the electrolyte maintains contact with the internal silver–silver chloride portion of the electrode. Although experience has shown it to be unnecessary to protect the electrode tips inside the smaller pilot plant equipment, the installation has been modified slightly in the larger production fermentors to minimize contact of the electrodes with solids in the fermentor. To accomplish this purpose, a 2-in. length of 6-in. pipe is welded to the inside wall to provide a protection ring, as shown in Fig. 4.

Fig. 5 shows the gland assembly in a pilot plant fermentor of 10-gal total capacity. Note that the water jacket has been cut away to allow installation directly through the inner wall of the

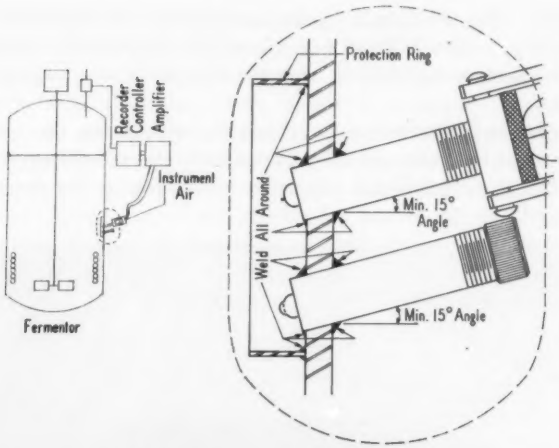

Fig. 4. Direct-entry installation

Fig. 5. View of pH gland installation in a 10-gal fermentor

fermentor. Fig. 6 shows a similar installation in a tank of 1,300-gal capacity. Here a third gland has been inserted to enable the use of a thermocompensator, a redox electrode or a second glass electrode.

The physical conditions to which the electrodes are exposed depend upon the size of the fermentor. In small fermentors, i.e. of 10-gal to 1,300-gal capacity, 20–25 p.s.i.g. air pressure is

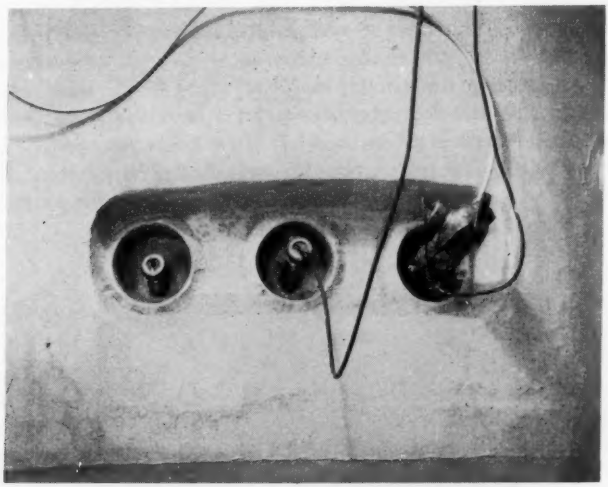

Fig. 6. View of pH gland installation in a 1,300-gal fermentor

maintained continuously on the reference electrode. It has been found necessary to replenish the electrolyte solution every 2–3 weeks. The electrodes are subjected to steam, hot water, and hot medium at 121 °C and 15 p.s.i.g. for 2–6 h each week. Once installed, the electrodes are not removed unless failure is suspected. Under these conditions of operation the average life of both glass and reference electrodes has been 6 months or more. The long life is due in part to the fact that the temperature of the electrode external to the fermentor is well below 121 °C during batch sterilization. Measurement of the temperature of the electrolyte solution

in the reference electrode has indicated that it does not rise above 85°C when the fermentor is heated to 130°C for 2 h.

In the 17,000-gal fermentors, the electrodes are placed 2·5 ft from the bottom of the vessel, and it is necessary to maintain 35 p.s.i.g. air pressure on the reference electrode to compensate for the fermentation-liquid height and the fermentor back-pressure to ensure a positive flow of electrolyte solution during fermentor operation. Location of the electrodes higher up the fermentor nearer the surface of the liquid would reduce the pressure requirement, but in our particular case would complicate the servicing problem. When the fermentor is not in operation, the pressure on

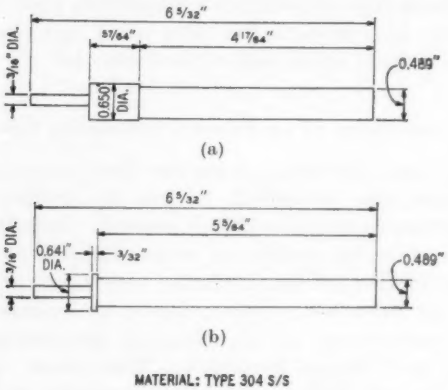

Fig. 7. Standard plugs for: (a) Glass electrode glands, and (b) reference electrode glands

the reference electrode is vented to atmosphere. Because of the severe vibration encountered during batch sterilization of the medium, it has been found necessary to fill the electrolyte reservoir to only one-half of its capacity in order to prevent electrolyte solution from splashing out and shorting the reference electrode. Under these conditions the reservoir has required filling once every 5–6 days. As in the pilot plant, the electrodes are subjected to steam, hot water, and hot medium at 121°C and 15 p.s.i.g. for 2–6 h each week. The electrodes are removed frequently during routine cleaning of the fermentors, and, because of this, mechanical

breakage is greater in the plant than in the pilot plant. When the electrodes are removed for any length of time, dummy electrodes made of stainless steel as illustrated in Fig. 7 are inserted to prevent debris of any type entering the glands.

Electrode life in the plant fermentors has been surprisingly good and, as one might expect, has improved as plant personnel have gained experience. For example, during the operation of the first 100 fermentation batches using this system, 12 glass electrodes and 15 reference electrodes were replaced. Electrode failures now range from 3 to 5 per 100 batches for both electrode types and even now the great majority of failures are due to accidental breakage. Our experience has demonstrated adequately that electrode life is sufficiently long in both the pilot plant and plant to justify installation directly in the wall of the fermentor.

Installation in an External Circulating System

Although electrode failure using the direct-entry technique has been minimal, the occasional failures do require unscheduled manual operations to maintain pH control. Installation of electrodes in an external circulating system has the advantage, particularly attractive to manufacturing operations, of enabling one to isolate the pH sensing system during the course of a fermentation. Consequently, an experimental circulating system was installed in a 17,000-gal fermentor. This system, which is illustrated in Fig. 8, consists of a pump, capable of being steam-sterilized and of running contamination-free, a pH gland flow assembly, and appropriate piping. The gland flow assembly, as illustrated in Fig. 8, consists of the two electrode glands welded to a short section of 1-in., schedule 40, type 316 stainless-steel pipe. This pipe in turn is welded at each end to a type 316 stainless-steel slip-on flange. Installed, the flanged connections are joined using a Teflon gasket. As in the direct-entry method of installation, the flow assembly must be installed at a minimum angle of 15° with the horizontal to ensure that the electrolyte maintains contact with the internal silver–silver chloride portion of the electrode.

This method of electrode installation has been demonstrated to have the following advantages:

(1) The electrodes can be removed, checked, and, if necessary, replaced without interrupting the fermentation.

(2) The location of the electrode assembly is not restricted and can be located for most convenient operation and service.

(3) The life of the electrodes is extended, since failure due to operating pressure, turbulence and vibration effects is minimized.

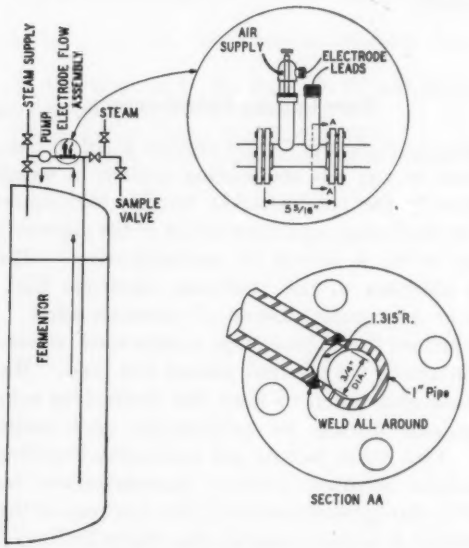

Fig. 8. External circulation installation

Use of Electrode Assembly for Monitoring pH

The electrode assembly as described has been used in a variety of pH monitoring systems. The simplest and most common application in the pilot plant has been its use with a standard pH meter, thus enabling continuous measurement of pH without the need for frequent sampling. More sophisticated systems have been built for use in both the pilot plant and plant for continuous reading and recording and for continuous recording and control.

In all of these systems the electrodes have given completely satisfactory service for periods up to 6 months or more without replacement.

The electrode assembly as described has also been used in monitoring fermentation oxidation–reduction potential. In this application, the O/R electrode (manufacturing code 19001) is used in the glass electrode gland in conjunction with a reference electrode assembly.

Pure Culture Considerations

The ability to maintain a pure culture is, of course, a foremost consideration in any pH monitoring system in which electrodes contact directly the fermentation broth. Serious consideration was given to the design and installation of the assembly to prevent its proving to be a source of contamination. Obviously, the electrolyte solution in the reference electrode had also to be considered as a potential source of contamination. In order to reduce the hazard from this source, a saturated potassium chloride solution containing 0·2 per cent phenol was used. Maximum precautions were also taken to keep the electrolyte solution free of micro-organisms during its preparation and addition to the electrode. Two other factors aid contamination-free operation: the electrolyte solution reaches pasteurization temperatures, i.e. 80°–85°C, during sterilization of the medium in the fermentor; and in addition, it is quite possible that the palladium junction acts as a bacteriological filter. This proposal is based on the fact that the annular opening around the junction of the Beckman industrial reference electrode, although an estimated 4–5 μ in size, is very much like a filter, owing to the sandblasting the palladium wire receives and to the fact that the rough surface becomes imaged on the glass as the glass cools in the manufacture of the electrode.[3]

The experience in our pilot plant and plant in a variety of fermentation processes and with a variety of micro-organisms has been that equipping fermentors with pH glands has not increased the incidence of contamination, but has provided valuable information and has made possible much better process control.

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A Preliminary Study of Depth Filtration at a Constant Rate*

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Summary. The constant-rate operation of a system utilizing asbestos filter-pads for the cold sterilization of a tissue culture medium is described. Filtration theory is briefly reviewed with regard to the constant-rate process, and the results of several data treatment methods are reported. The standard law of Hermans and Bredée[4, 5] best describes the constant-rate data. Computation of the data, when plotted by the standard-law method, results in two straight lines divided by a transition zone. The second region is characterized by a slower plugging rate and a plot of the standard-law slopes versus the initial filtration pressures shows a linear relation. The constant-rate method is compared with the constant-pressure method and is found to yield equivalent overall filtrate rates at lower operating pressures.

The observed filtration behaviour is discussed, and available data are presented to indicate the sterilization capability of the filter.

Introduction

The deleterious effects of heat sterilization on certain biological media make the use of an alternative sterilization method necessary. Filtration through Seitz asbestos pads has been proved an effective method for the sterilization of a tissue culture media which contains heat-labile biological components. In a previous work[1] we presented theoretical aspects of sterilization of a tissue culture medium by constant-pressure filtrations. This paper describes the extension of that work to the field of constant-rate processes.

Information on the possible sterilization by constant-rate filtration was considered necessary because the constant-rate process is more compatible with desired low initial operating pressures. These low initial pressures were recommended by the filter-pad

* This paper was presented before the ACS meeting held in New York, September 1960.

manufacturer* to assure retention of bacteria. Filtration theory is briefly reviewed with respect to constant-rate processes, and preliminary results and tentative conclusions which may be drawn at this time are presented.

The Seitz No. 200 laboratory filter press with asbestos filter-pads was used in our earlier work and formed the basis also for this investigation. This type of filter is applicable to the recovery of fermentation products in cases where the removal of micro-organisms is required. Such a system is of especial value where either a sterile or a highly polished product is deemed essential. Operation of such a filter at constant rate would be desirable in conjunction with continuous fermentation or any other continuous-process system. In addition, process design calculations would be simplified for systems using constant-rate filtration.

Most investigators have only given consideration to the theory of constant-pressure filtrations. Thus, the constant-rate process, which is less frequently used, is probably not as well understood as are constant-pressure filtrations.

Similarly, most investigators have given consideration to the theory of so-called cake filtrations, operations which are common in the chemical industry. In such operations, the liquid filtered normally contains a relatively large amount of solids. These solids are deposited on a cloth or other suitable filter material and gradually form a filter-cake which is itself the effective filtering medium. Carman [2] was probably the first to give a complete and correct mathematical explanation of the theory of this type of filtration in terms of the experimental data. Ruth *et al.*[3] likewise pioneered in the field of filtration theory in developing the well-known Ruth Equation. However, Hermans and Bredée [4, 5] first derived mathematical relationships for types of filtrations other than those in which a cake is formed. They studied such filtrations as those involving removal of material present in amounts less than 100 p.p.m.

Of available theory tested in our earlier work on sterilization of tissue culture media with a constant-pressure system, the standard law of Hermans and Bredée [4, 5] best fitted the data. This law was derived by assuming filter pores of constant length which decrease in diameter as particles are adsorbed on the pore walls. In a

* Republic Seitz Filter Corp., Newark, N.J.

similar manner, these authors derived a cake-filtration law by assuming pores of constant diameter which increase in length during cake build-up.

Grace[6] and other authors confirmed the usefulness of the standard law in describing systems in which there is a relatively small amount of particulate matter in the feed. Thus, we expected that the sterilization of a liquid containing a presumably small concentration of contaminants would reasonably follow the standard law.

Grace[6] showed that the filtration laws derived by Hermans and Bredée[4, 5] can be reduced to two differential equations, one describing constant-pressure filtrations and the other describing filtrations at constant rate.

The general differential equation for filtrations at constant pressure is:

$$d^2t/dV^2 = K(dt/dV)^n$$

where t = time, V = volume of filtrate, K = a constant, and n = a constant.

For filtrations at constant rate, the general differential equation is:

$$d(\Delta P)/dV = K(\Delta P)^n$$

where ΔP = differential pressure.

In both cases the value of the exponent n defines the mode of filtration occurring, while the value of K depends on the liquid being filtered, the filtering medium, and the conditions of filtration.

For the common cake-type of filtration the value of n is zero. For the standard-law mode of filtration the value of n is $\frac{3}{2}$. Integration of the constant-rate differential equation with $n = \frac{3}{2}$ yields the equation:

$$(\Delta P_0/\Delta P)^{1/2} = 1-(K_sV/2)$$

where ΔP_0 = initial differential pressure, V = volume of filtrate, ΔP = pressure after passage of volume V, and K_s = standard-law plugging constant.

This equation represents the standard law for filtrations at constant rate. If this law be followed, a linear relationship would exist between the volume of filtrate V and the square root of the ratio of initial pressure to instantaneous pressure.

Experimental Method

The liquid filtered experimentally was a tissue-culture medium consisting of 89 per cent Eagle's[7] medium, 10 per cent bovine serum, and 1 per cent chick-embryo slurry. The serum and embryo slurry were previously filtered twice to ensure absence of pleuropneumonia-like organisms.

Fig. 1 shows the experimental apparatus. A standard 100-ml Seitz No. 6 laboratory filter had been modified for use as a pressure filter by soldering a cover plate with hose connection to the top of the filter. The sterilizing medium was Seitz acid-washed asbestos pads of 20 mμ porosity.

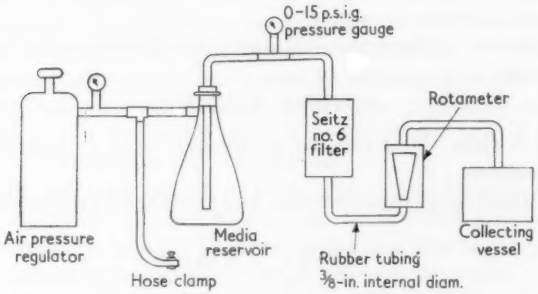

Fig. 1. Experimental apparatus for constant-rate filtrations

Before each filtration run was begun, the rotameter and connecting tubing were filled with water to minimize the lag in flow-rate readings. The air-pressure regulator was adjusted and the bleed hose was throttled to obtain the desired initial pressure. As soon as the flow rate steadied, the position of the float was marked on the rotameter scale. The pressure was adjusted to maintain a constant filtrate rate and the pressure was recorded at regular intervals. Runs were made from initial pressures of 1, 2 and 3 p.s.i.g., which represented flow rates of 0·42, 0·54, and 0·64 ml/min cm^2.

Results

Computations from the preliminary data were plotted according to the four basic filtration laws derived by Hermans and Bredée.[4, 5]

These are the standard law, the cake law, the intermediate law, and the law of complete blocking. The complete-blocking law was of no use in describing the system. Unexpectedly, however, the cake law, the standard law and the empirical intermediate law all fitted the data reasonably well.

Fig. 2 shows the data from a typical constant-rate filtration run plotted according to the cake-law method. This law postulates filter pores of constant diameter which increase in length as a cake builds up on the surface of the filtering medium. The resulting plot can be divided into two linear regions divided by a transition zone. The lesser slope of the final region indicates a slower rate of plugging of the pores of the filter.

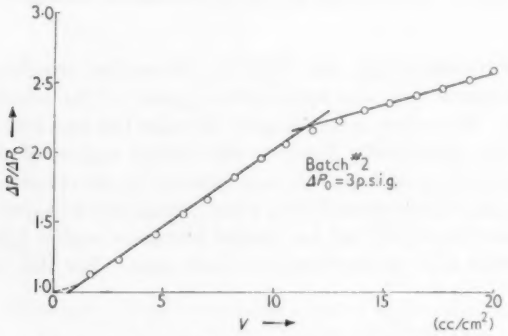

Fig. 2. Cake-law plot for a constant-rate filtration

Fig. 3 shows the standard-law plot of the same data that is represented by a cake-law plot in Fig. 2. All constant-rate data, when plotted by the standard-law method, resulted in two straight lines separated by a transition zone. For each run the slopes of both linear regions were measured.

The data from all the filtration runs were plotted by both the cake-law and the standard-law methods. In a majority of the constant-rate filtration runs to date, the data could be equally well represented by both the standard-law plot and the cake-law plot. This supports the view of Gonsalves[8] that the adherence of data to a particular filtration law does not in itself prove the theoretical mechanism on which the law is based.

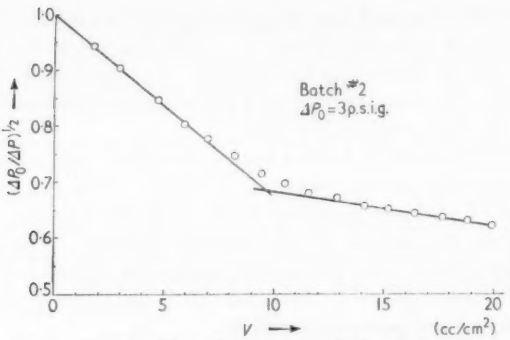

Fig. 3. Standard-law plot for a constant-rate filtration

The intermediate law, for which no theoretical mechanism has been postulated, was also applicable to most of the constant-rate filtrations. However, in some cases the cake law and intermediate law did not adequately describe the initial region of filtration. Variation from these laws was represented by an erroneous intercept when the experimental data were extrapolated to zero volume. The standard-law plot for the initial filtration region intercepted at the correct unit pressure ratio in each case. For this reason we

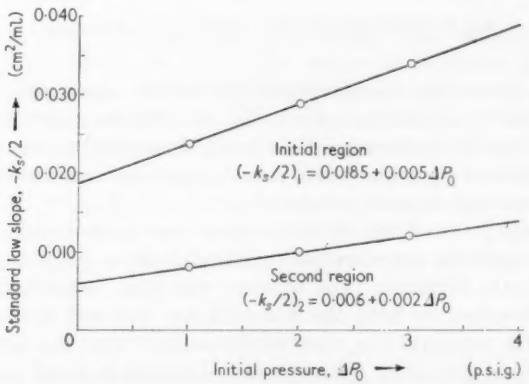

Fig. 4. Correlation between standard law slopes and ΔP_0

chose the standard-law method of Hermans and Bredée[4, 5] for the analysis of our filtration data. In our previously reported work, we noted also some instances in which the cake law expressed the data as well as did the standard law.

The average values of the slopes of the standard-law curves obtained were plotted as a function of the initial pressure, ΔP_0. The resulting relations were linear as shown in Fig. 4.

The transition zone between the two standard-law regions usually occurred after the passage of 7 ml and before the passage of 10 ml of filtrate/cm² of filter area. For convenience in analysing the data, the point of intersection between the two straight-

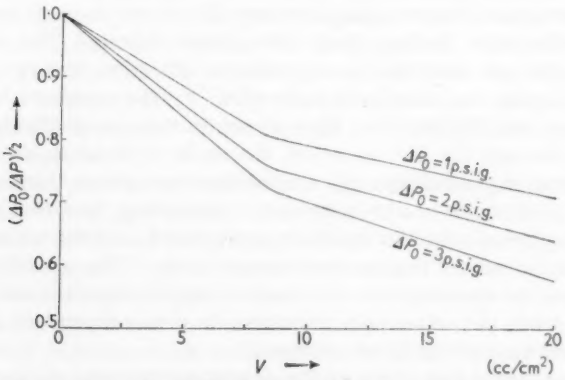

Fig. 5. Composite standard-law plots based on average values of experimental results

line plots was determined for each filtration. These values were averaged for each pressure and plotted versus the initial pressures. However, no relation was found between these points of intersection and initial pressure. The average value for all runs was $8{\cdot}4 \pm 1{\cdot}65$ ml of filtrate/cm² of filter area.

Using the average standard-law slopes for runs at each initial pressure and the average transition point for all runs, the composite standard-law plots shown in Fig. 5 were determined. These curves can be used to predict the pressure reached at any point in a constant-rate filtration.

Discussion

The two different regions determined by the standard-law plots may be compared to results obtained by Grace[6] with a somewhat similar system. He investigated the performance of filter media commonly used for the clarification or 'polishing' of liquids. The pore sizes involved were larger than those contained in the Seitz S-1 T sterilizing pads. The ratio of pore length to pore diameter is much greater for the Seitz pads. This is of particular importance in regard to the adsorption of minute particles on the pore walls. Notwithstanding this difference, the filtration mechanisms may be considered comparable for the two systems.

Grace[6] found that constant-pressure filtrations through clarifying media were broken down into three regions. The initial region did not conform to any known filtration theory. The second region was adequately described by the standard law of Hermans and Bredée.[4, 5] After a certain volume of filtrate had passed through the pad, however, the mode of filtration changed. The filtration passed through a transition zone into a third region which conformed to the cake law. According to Grace,[5] this initial region of cake-law filtration represented cake filtration on a microscopic rather than a macroscopic scale. The specific cake resistance he computed on the basis of superficial filter area was 2 to 30 times the actual cake resistance, and was dependent on the pore structure of the filter medium.

The standard-law plots of the constant-rate data showed two linear regions divided by a transition zone. The cake-law plots were linear in the second region but in some cases the cake law could not be applied to the first region. The constant-rate cake-law equation derived by McCabe and Smith[9] from the Kozeny–Carman equation indicates a linear relation between the log of the pressure increase and the log of the time. This relation fitted the data very well over the second region but did not provide linearity over the first region. Since the standard law was adequate for both regions, it was more convenient to apply it to the entire filtration.

The transition between the two filtration regions, evident in the standard-law plots of constant-rate data, was not detected in earlier filtrations at constant pressure. However, the filtrations

at constant pressure were terminated after the passage of only about 10 ml of filtrate/cm^2 of filter area. The constant-rate tests were extended to a filtrate through-put of as much as 20 ml/cm^2.* Therefore another test at constant pressure was performed and was extended to 20 ml/cm^2. There was a transition zone from about 10 ml to 14 ml/cm^2. The change in slope was not as great as was observed in the constant-rate filtrations. However, additional data are required to verify the degree of change.

The filtration mechanism causing the transition to a slower plugging rate could not be determined from the data collected in this investigation. The fact that the standard law described the data in the second region as well as in the first does not necessarily rule out the formation of a microscopic cake. Such a cake could be used to explain the decrease in plugging rate. However, it is improbable that the liquid filtered in these tests contained particles of sufficient size to form such a microscopic cake, since the biological components of the medium had already undergone two prior filtrations.

Another possible explanation of the change in plugging rates might be found in the shearing away of adsorbed particles. The narrowing of the zone of primary adsorption and shearing away of adsorbed particles by increased pressure have been reported by Ferry[10] in a study of ultrafiltration. At a certain point in the constant-rate filtration, the filtration pressure and thickness of adsorbed particles reach sufficient magnitudes to shear off. The plugging rate might then be expected to decrease. The data from this investigation show the transition to occur in the range of 7–10 ml filtrate/cm^2 regardless of initial filtration pressure. This would indicate approximately the same depth of adsorbed particles on the pore walls in each case. The differential pressure at the transition point was about 1·5 p.s.i.g. starting from an initial pressure of 1 p.s.i.g. However, from an initial pressure of 3 p.s.i.g. the pressure during the transition period was around 5·5 p.s.i.g.

The data obtained in this constant-rate investigation and data from the previous constant-pressure work were used for the plot of filtrate volume versus time shown in Fig. 6. The constant-rate

* These particular through-puts do not reflect any experimentally determined maximum through-puts before a breakthrough of contaminants may occur.

run from an initial pressure of 2 p.s.i.g. is of course represented by a straight line. The constant-pressure curve was calculated from the general equation for constant-pressure sterilizations that was developed in our previous work.[1] Since experimental results did not extend past a filtrate volume of 10 ml/cm^2, the dashed line above that point represents an extrapolation of the experimentally determined equation.

The constant-rate line and the 4 p.s.i.g. constant-pressure line intersect at a filtrate volume of 8 ml/cm^2 after 15 min. However,

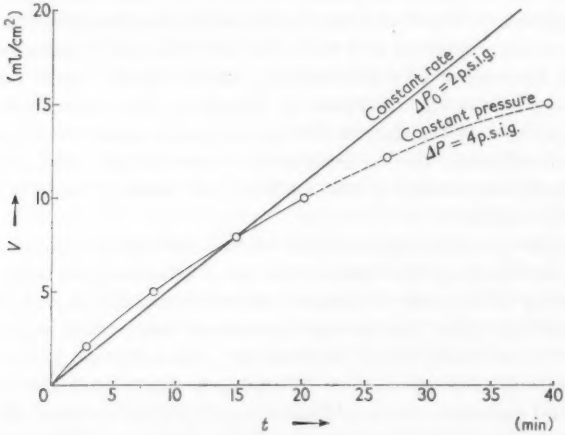

Fig. 6. Volume of filtrate *versus* time; comparison of constant rate and constant pressure

the constant-rate filtration has only reached a pressure of 3·5 p.s.i.g. at this point. This is still lower than the constant pressure of 4 p.s.i.g. Thus, for the system here investigated, the constant-rate process is preferred to constant pressure because lower pressures yield a given filtrate volume in a given time. However, the volume of filtrate that may be passed at constant rate before a breakthrough of contaminants occurs has not as yet been determined.

Routine use of the filter for medium sterilization, in which a constant-rate process was involved for 6 runs, has shown that

sterile filtrates may be achieved for through-puts of up to 6·1 ml/cm^2 in such a process. Over 60 runs with constant pressure or semi-constant rate (in which a low initial pressure was increased in one or more steps during filtration) have been made without a failure. Through-puts as high as 20 ml/cm^2 have been obtained without contaminants appearing in the filtrate. With the use of three stages in series in the filter press, 60 ml of a bacterial medium/cm^2 of pad area were successfully sterilized.

Imperviousness of the pads to bacteria and pleuropneumonia-like organisms was tested with known concentrations of these organisms. Details concerning these tests and our experiences with the filter from the standpoint of sterilizing media are intended for publication later. Suffice it to say at present, the pads were found to withhold *Serratia marcescens* at a concentration of 10^8 cells/ml. Pleuropneumonia-like organisms, which are known to be deformable, were found to pass in one test of three but only when present in massive amounts and near the end of the filtration. Such concentrations are very unlikely to be found in processing any media or sera normally encountered.

All routine filtrations at constant pressure have proved to result in a sterile filtrate. Although the maximum safe through-put depends on concentrations of micro-organisms and adsorbable materials in the feed liquid as well as operating pressures, the constant-rate process should be at least as effective as the constant-pressure process when the desirability of low initial operating pressures is considered.

Conclusions

Constant rate filtration of a tissue-culture medium through Seitz asbestos sterilizing pads is adequately described by the standard law of Hermans and Bredée. A transition to a slower plugging rate occurs during the constant-rate filtration.

The constant-rate method allows the low initial filtration pressure recommended for absolute sterility of the filtrate. Over the range tested, similar filtration pressures give better overall filtrate flow than those of the constant-pressure method.

Although the data presented are preliminary in nature, they

have provided a better understanding of sterilization by constant-rate filtration and could serve as the basis for a more extensive investigation of the constant-rate process.

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A Method for the Preparation of Undigested Bacterial Cell Walls*

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Summary. The combination of low-temperature disintegration with gradient and pile centrifugation makes possible the preparation of bacterial cell walls under conditions preventing digestion.

Introduction

In general, bacterial cell walls are prepared by the method described by Salton [1] or by various modifications of this method. The procedures involve the following steps: (1) The cells are disintegrated by shaking with glass beads. (2) The cell walls are separated from the glass beads, non-disintegrated cells and somatic material by differential centrifugation. Since conventional differential centrifugation is not sufficiently selective to permit the separation of cell walls from intact cells, the bacteria must be almost completely disintegrated in advance. Some kinds of cells are very difficult to disintegrate by mechanical shaking, and treatments exceeding 1 h are often necessary. [2] During the disintegration, bacterial cell walls are exposed to the action of cellular proteinase, ribonuclease and other enzymes under rising temperatures. This may cause a breakdown and denaturation of the material. Under the circumstances it is reasonable to assume that cell walls obtained by such methods have different activities from those of 'undigested' cell walls.

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Recently, cell walls which might be considered as 'undigested' were obtained from *Bordetella pertussis* and their biological activity was discussed.[3] However, the technique used is not a suitable routine method for the preparation of other kinds of bacterial cell walls.

In order to investigate the biological properties of 'undigested' staphylococcal cell walls, a new general method for the preparation of bacterial cell walls was developed. This method involves the following steps: (1) bacterial cells are disintegrated in the 'X-press'[4] under high pressure at *ca.* −25°C; (2) disintegrated cells are washed several times with phosphate–citrate buffer and with 1 M KCl after centrifugation; and (3) cell walls are separated from non-disintegrated cells and other cellular material by density gradient centrifugation[5] and by pile centrifugation. By this method cell walls which may be considered as 'undigested' were obtained from *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus megaterium*, *Escherichia coli* and *Bordetella pertussis*.

Methods

1. *Microbiological Material Used.* A virulent *Staphylococcus aureus* strain,[6] phage type 80/81 was cultivated in 100–200 l. batches of the medium described by Sall, Mudd and Taubler.[7] The bacteria were grown for 24 h at 37°C under vortex aeration in a 'biotechnical work unit'[8] and were then harvested on Sharples centrifuges. The bacterial paste was stored in the frozen state until used.

Bordetella pertussis cells were obtained from a 500-l. batch culture prepared as described elsewhere,[9] and *Str. faecalis*, *B. megaterium* and *E. coli* cells were obtained from cultures grown as described by Edebo.[10]

2. *Method of Disintegration.* The 'X-press' method described by Edebo[4] was used. Disintegration was carried out in a large press of about 30-ml capacity by passage three times through a small orifice at −25°C.

3. *Method of Density Gradient Centrifugation.* A linear density gradient was prepared by a procedure described by Ribi *et al.*[5] in a nitro-cellulose centrifuge tube (Servall tube, 50 ml). For the first separation, a tube which contained 40 per cent sucrose in

1 M KCl at the bottom and a disintegrated cell suspension (less than 1 g dry weight) in an upper layer (Fig. 1a), was centrifuged in a horizontal rotor (International No. 269) at 2,500 rev/min for 60 min at 0°C. The cell walls, which formed a layer at the middle of the tube, were separated by means of a syringe. They were then purified by centrifugation at 2,500 rev/min for 60 min in a tube which held a linear gradient of 30 per cent sucrose in 1 M KCl. (Fig. 1b).

4. *Method of Pile Centrifugation.* The disintegrated cell suspension was deposited on the surface of a homogeneous medium of suitable density contained in a centrifuge tube. It was then spun in the horizontal rotor mentioned above.

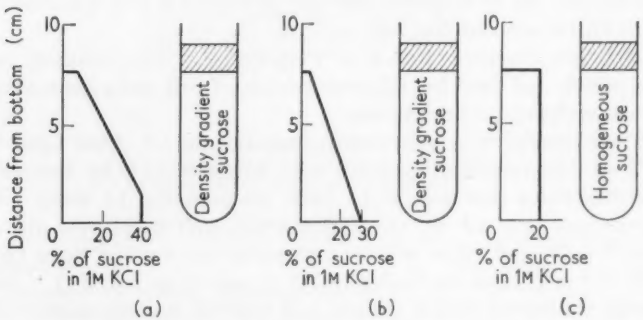


Fig. 1. Centrifuge tubes for density gradient centrifugation (a and b), and for pile centrifugation (c). Disintegrated cell suspension (7–10 ml) was piled on the surface of a sucrose solution, and centrifuged by an International No. 269 rotor at 2,500 rev/min at 0°C

The sedimentation velocity of the cells (v_1) and of the cell walls (v_2) can be expressed by the following equations:

$$v_1 F_1 = (d_1 - d_0) V_1 g$$
$$v_2 F_2 = (d_2 - d_0) V_2 g$$

(d_0 = density of the medium, d_1 = density of the mean cell, d_2 = density of the mean cell wall, V_1 = volume of the mean cell, V_2 = volume of the mean cell wall, F_1 = frictional force at a cell-per-unit sedimentation rate, F_2 = frictional force at a

cell-wall-per-unit sedimentation rate, g = the force of gravity in the centrifuge field.)

Considering the fact that cells have larger volumes than cell walls, and that cells are more globular than cell walls, V_1/F_1 is, in general, greater than V_2/F_2. Accordingly,

$$v_1/v_2 > (d_1-d_0)/(d_2-d_0).$$

If the density of the medium is close to that of the cell walls, v_1 is much greater than v_2. An effective separation of cell walls from non-disintegrated cells can then be realized.

For cell-wall preparations, 20 per cent sucrose in 1 M KCl was used as the homogeneous medium (Fig. 1c), and centrifugation was carried out at 2,500 rev/min for 30 min at 0°C in a horizontal rotor (International No. 269).

5. *Whole Process of Cell-Wall Preparation.* The complete process which was used for the preparation of cell walls from *Staph. aureus* is shown in the diagram.

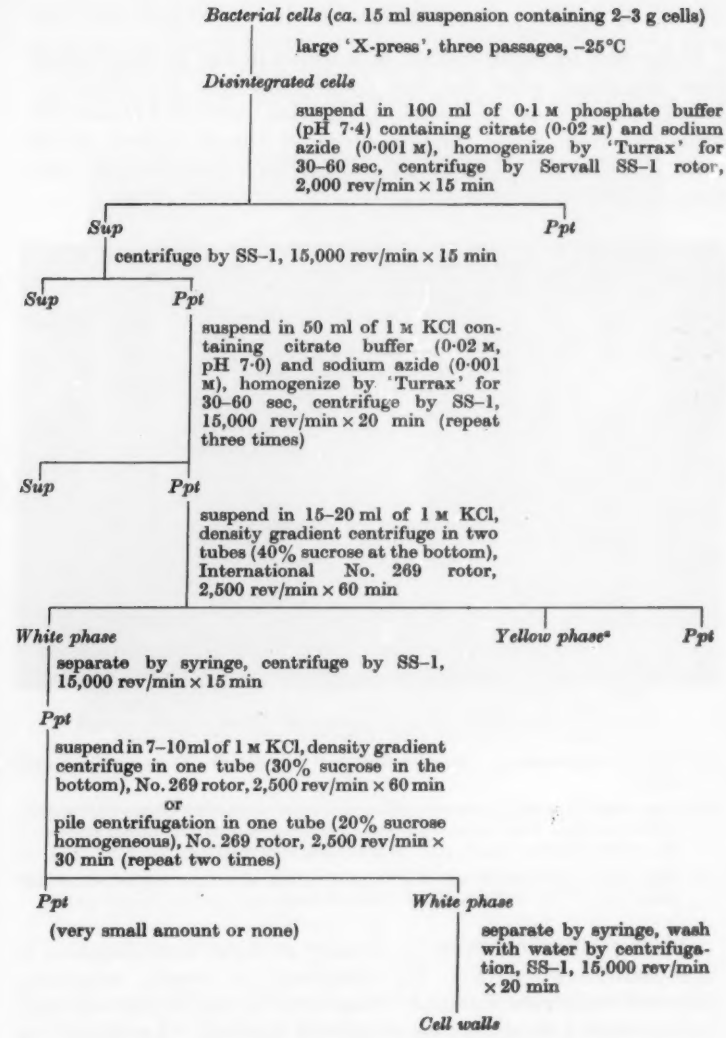
The bacterial cells were disintegrated in the 'X-press', and the bulk of the non-disintegrated cells was removed by low-speed centrifugation (Servall SS–1, 2,000 rev/min for 15 min). The particulate content of the supernatant (cell walls, non-disintegrated cells, and other cellular materials) was washed three times with 0·1 M phosphate buffer (pH 7·4) and with 1 M KCl. Both media contained 0·02 M citrate and 0·001 M sodium azide. The washings were carried out through homogenization by means of a 'Turrax' mixer* (10^4 rev/min) and centrifugation at 15,000 rev/min for 20 min (Servall SS-1 rotor).

The precipitate was fractionated by density gradient centrifugation in a tube which had 40 per cent sucrose at the bottom. The cell-wall fraction was separated by a syringe, to be further purified either by density gradient centrifugation in a tube which had 30 per cent sucrose at the bottom, or by pile centrifugation.

Purified cell walls were washed three times with distilled water by centrifugation (Servall SS-1 rotor, 15,000 rev/min for 20 min) and were then stored at −20°C.

All the procedures were carried out under efficient ice cooling.

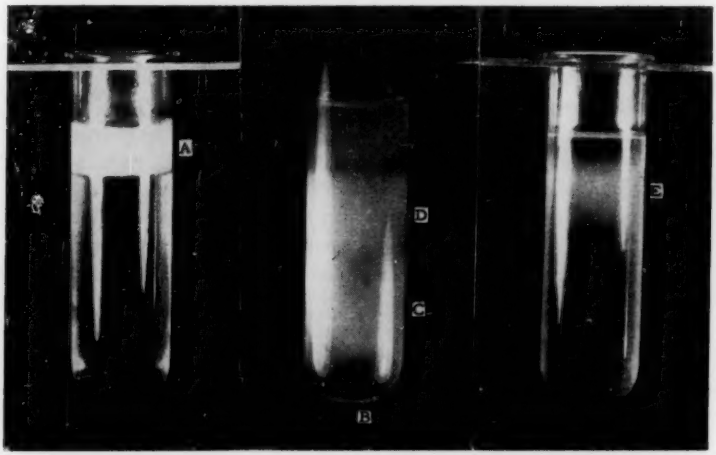
* High-speed homogenizer (Janke & Kunkel, K.G., Sweden).


Bacterial cells (ca. 15 ml suspension containing 2–3 g cells)
large 'X-press', three passages, –25°C
Disintegrated cells
suspend in 100 ml of 0·1 M phosphate buffer (pH 7·4) containing citrate (0·02 M) and sodium azide (0·001 M), homogenize by 'Turrax' for 30–60 sec, centrifuge by Servall SS–1 rotor, 2,000 rev/min × 15 min
Sup
Ppt
centrifuge by SS–1, 15,000 rev/min × 15 min
Sup
Ppt
suspend in 50 ml of 1 M KCl containing citrate buffer (0·02 M, pH 7·0) and sodium azide (0·001 M), homogenize by 'Turrax' for 30–60 sec, centrifuge by SS–1, 15,000 rev/min × 20 min (repeat three times)
Sup
Ppt
suspend in 15–20 ml of 1 M KCl, density gradient centrifuge in two tubes (40% sucrose at the bottom), International No. 269 rotor, 2,500 rev/min × 60 min
White phase
Yellow phase*
Ppt
separate by syringe, centrifuge by SS–1, 15,000 rev/min × 15 min
Ppt
suspend in 7–10 ml of 1 M KCl, density gradient centrifuge in one tube (30% sucrose in the bottom), No. 269 rotor, 2,500 rev/min × 60 min
or
pile centrifugation in one tube (20% sucrose homogeneous), No. 269 rotor, 2,500 rev/min × 30 min (repeat two times)
Ppt
(very small amount or none)
White phase
separate by syringe, wash with water by centrifugation, SS–1, 15,000 rev/min × 20 min
Cell walls

* This fraction was separated by syringe and purified by density gradient centrifugation in a tube containing 40% sucrose at the bottom.

Results

In the case of *Staph. aureus*, as is shown in Fig. 2b three phases were recognized after the first density gradient centrifugation. It was found that the yellow precipitate contained intact cells while the upper white fraction consisted almost entirely of cell walls. The middle yellow phase consisted of cells which were Gram-negative, but fuchsine-positive and optically dense.



(a) (b) (c)

Fig. 2. Centrifuge tube before and after centrifugation

(a) Before centrifugation. Disintegrated cell suspension (A) is seen on upper layer.

(b) After density gradient centrifugation in a tube containing 40 per cent sucrose at the bottom. Three phases are seen. B is a precipitate of intact cells. C is the yellow fraction (see text). D is the cell wall fraction.

(c) After pile centrifugation in a tube containing 20 per cent sucrose. Single phase (E), which consists of cell walls, is seen.

After purification twice by density gradient centrifugation or pile centrifugation (Fig. 2c) completely or nearly completely pure cell walls were obtained. Less than 100 mg of pure cell walls were obtained from 2 g (dry weight) of bacteria. Assuming that the cell-wall portion of *Staph. aureus* is about 20 per cent, the yield was less than 25 per cent. This poor yield was due to the

fact that staphylococcal cells are extremely difficult to disintegrate by the freeze-pressing method employed. The yields were particularly small when the water content of the disintegrated cell paste was low, as in material obtained directly from the

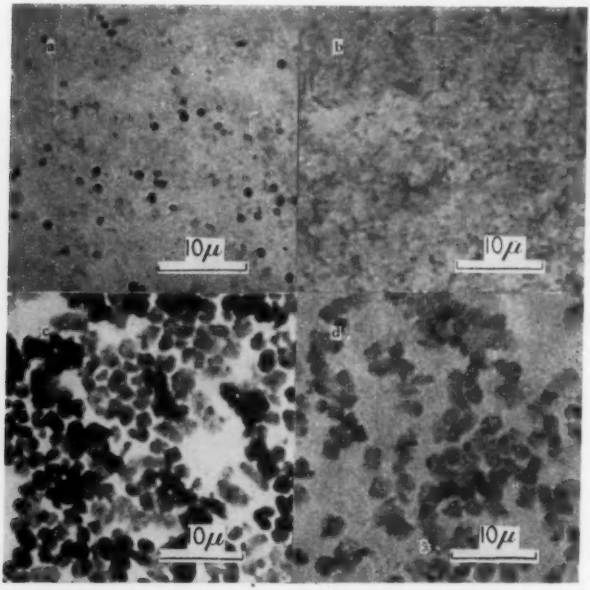


Fig. 3. Micrograph of cells and cell walls during the course of the cell wall preparation

(a) *Staphylococcal* cells and cell walls after density gradient centrifugation. Small amounts of intact cells are seen.

(b) Purified *Staphylococcal* cell walls.

(c) *Bacillus megaterium* cells and cell walls before density gradient centrifugation. Many intact cells are seen.

(d) Purified *Bacillus megaterium* cell walls.

Sharples centrifuge. When a paste having the consistency of porridge or a thick slurry was frozen the result was better.

Cell walls from other kinds of Gram-positive and Gram-negative bacteria, such as *Str. faecalis*, *B. megaterium*, *E. coli* and *Bordetella*

pertussis, were prepared by the method described above with only minor modifications. In all the cases, with the exception of *Str. faecalis*, two phases, i.e. non-disintegrated cells at the bottom of the centrifuge tube and cell walls in a middle phase, were recognized after the first gradient density centrifugation. In the case of *Str. faecalis*, the same three phases were obtained as with *Staph. aureus*. A layer of Gram-negative optically dense particles was obtained also in this case.

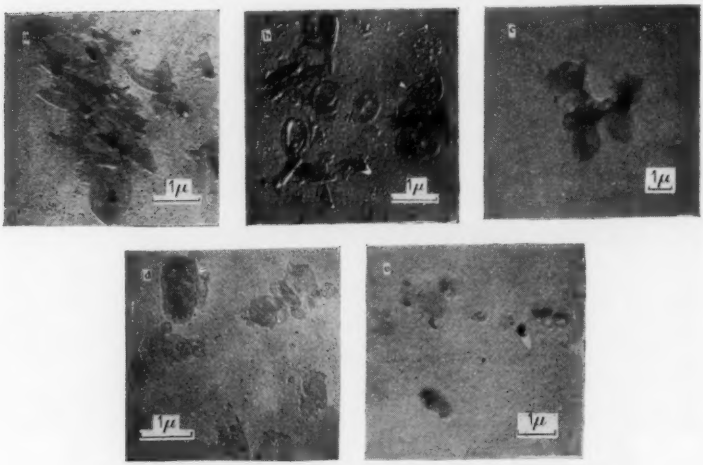


Fig. 4. Electron micrographs of cell walls
(a) *Staphylococcus aureus.*
(b) *Streptococcus faecalis.*
(c) *Bacillus megaterium.*
(d) *Escherichia coli.*
(e) *Bordetella pertussis.*

The yield of the cell walls was better than 50 per cent in the cases of *B. megaterium*, *E. coli* and *Bordetella pertussis*.

Micrographs of cells and cell walls during the course of cell-wall preparation are shown in Fig. 3.

Electron micrographs of the cell walls obtained from these micro-organisms are shown in Fig. 4.

Discussion

It is obvious that particles which have different densities can be separated by density gradient centrifugation if agglutination effects do not disturb the situation. Actually, cell walls could be separated effectively from intact cells and other particles by this method.

In the case of pile centrifugation, it is not necessary to centrifuge until a sedimentation equilibrium is reached. The centrifugation time can therefore be shorter than with gradient centrifugation. The former method is also simpler than the latter. However, the three fractions which could be separated by density gradient centrifugation could not be separated effectively by direct pile centrifugation. This method was only utilizable for the purification of cell walls after a preliminary separation by gradient centrifugation had been achieved.

In the process of preparation, cell walls are exposed to cellular enzymes only for a short time at 0°C, and they are not exposed to any drastic treatments. Cell walls obtained by the present method may be considered as 'undigested'.

The biological properties of cell walls obtained from *Staph. aureus* will be reported in separate papers.

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Miso—I. Preparation of Soybeans for Fermentation

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Summary. The preparation of soybeans for fermentation is a very important part of the process for making miso, a traditional Japanese food. In general, soybeans from the United States are smaller and more pigmented than Japanese soybeans. Twenty-eight American varieties, four Japanese varieties and one Chinese variety have been studied with respect to absorption of water and cooking. A variety of soaking and cooking conditions have been investigated. Some U.S. varieties absorb water and cook very unevenly. Unevenness in cooking is not associated with the size of the beans within a variety.

Introduction

Miso is a Japanese food prepared by the fermentation of mould rice, soybeans and salt. Although miso has been prepared for at least a thousand years in Japan, only two general accounts have been published regarding this food in the United States.[1, 3] This report concerns studies of the preparation of the soybeans for fermentation. A general account of the fermentation conditions, uses, equipment, process, etc. employed in the manufacture of miso will shortly be published by the authors.

Koji (mould rice) is prepared by inoculating cooked rice in shallow trays with strains of *Aspergillus oryzae* and by allowing the mould to grow over the rice kernels. The mould rice serves as a source of enzymes for the second part of the fermentation. At the same time, whole soybeans are prepared for fermentation by soaking in water and cooking.

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Next, salt, mould rice and cooked soybeans are ground together and inoculated with a small amount of miso from a previous fermentation. After thorough mixing of the ingredients, the material is packed into suitable containers and allowed to undergo a long period of fermentation from room temperature to 35–40°C without aeration. The fermented substrate is allowed to age for several months at room temperature and then is ground into a uniform product of the consistency of peanut butter and often with about the same colour. Finished miso is then packaged for use as a food and sold in the range of 9 to 13 cents a pound according to Smith.[3] Miso is principally used to make soup that takes the place of our cereal for breakfast. About 13 million bushels of soybeans are used to make miso each year; annual production amounts to 974,000 metric tons.

Our interest in this fermentation arose because Japan imports soybeans principally from the United States. For example, Hayashi[2] states that in 1958 676,997 metric tons of soybeans were imported into Japan from the United States. Plant breeding has considerably modified United States soybeans from the ancestral stock brought from the Orient. Japanese food processors report that U.S. soybeans do not absorb water or cook as readily as competitive beans, that fermentation is slower, and that the colour, flavour and texture of miso made from U.S. beans are not as satisfactory as those of miso made from their own or Chinese beans. This study was undertaken in order to improve the miso fermentation using American soybeans.

Materials and Methods

The various U.S. soybean varieties were obtained through the co-operation of the U.S. Regional Soybean Laboratory, Urbana, Illinois, and Mr. L. E. West of the Farmer City (Illinois) Grain Company. The moisture content of U.S. soybeans was about 7 per cent. Japanese soybeans, shipped in burlap bags through the kindness of the Japanese–American Soybean Institute of Tokyo, were commercial grade No. 2 and had a moisture content of almost 10 per cent on arrival at Peoria. Moisture content is important, for it is related to the velocity of water absorption during soaking.

Each of the experiments involving the soaking of soybeans was done with 10-g lots in Petri dishes. Distilled water was used since the hardness of water affects not only the softness of cooked soybeans, but also the miso fermentation. After soaking, the soybeans were cooked in a special 30-l. stainless-steel cooker. The cooker is shown in Fig. 1.

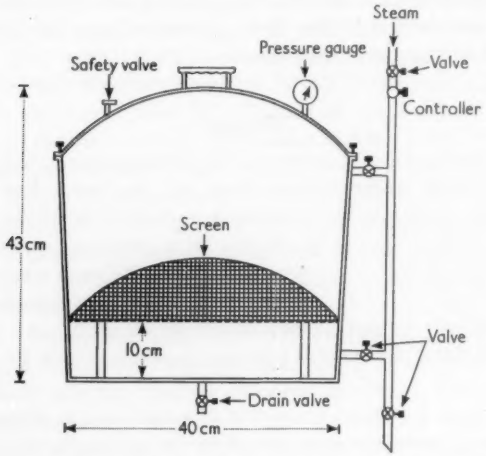

Fig. 1. Soybean cooker used to cook soybeans prior to the miso fermentation

A modified, standard, home-canning cooker, similar to those used in Japan, was installed in place with a drain. Steam was connected at two points, at the top and bottom, and a perforated stainless-steel basket was placed inside the cooker about 10 cm above the bottom. The basket was lined with cheese-cloth and then filled with soaked soybeans for cooking.

In cooking, it is very important first to allow the steam to flow into the cooker from the top line for about 5 min with the drain open and the bottom steam-line closed. The purpose of this step is to steam the beans, remove the undesirable soybean odour and taste, and to allow the flavour and odour to escape. Following this step, the top line is closed, the bottom line is opened and the

drain is almost closed. The cooking pressure is regulated in the steam line leading to the cooker. Soaking and cooking times are given in the tables below, under 'Results'.

A Penetrometer, manufactured by the Precision Scientific Company,* was employed to measure the hardness of steam-cooked soybeans. This apparatus is used to determine the firmness of bread and normally uses a weight of 100 g. Since cooked soybeans are harder than bread, an additional weight of 100 g was added. The data presented are the figures read directly from the Penetrometer.

Results

In the preparation of soybeans for fermentation, they must be soaked in cold water before they are cooked. For example, Sendai miso is prepared by placing soybeans in water at a temperature of 12–15°C for 15–18 h. In a preliminary experiment, two varieties of U.S. soybeans from the 1958 crop were soaked in distilled water at 25°C. They were extreme examples of U.S. soybeans which do not absorb water at all well. At the end of 24 h and 7 days the results were as shown in Table I.

Table I. Ability of two U.S. soybeans to absorb water

Variety	Total no. of beans	At 24 h	%	At 7 days: Absorbed water only, %	At 7 days: Germinated only, %	At 7 days: Germinated and grew, %
Harosoy	99	Beans absorbing water	75	18	8	49
		Beans not absorbing water	24	10	3	12
Comet	102	Beans absorbing water	48	13	0	35
		Beans not absorbing water	52	5[a]	3	31

[a] Even after 7 days, 13 per cent did not absorb water.

* The mention of products does not imply endorsement or recommendation by the Department of Agriculture over other products of a similar nature not mentioned.

The table shows that some varieties do not absorb water rapidly and consequently cannot be cooked. The two varieties, Harosoy and Comet, are of interest because they have a common hereditary background. Harosoy was made from the variety, Mandarin, back-crossed, and Comet, from Mandarin crossed with Pagoda.

Twenty-two representative varieties from the 1958 U.S. crop of soybeans were studied as to appearance of their hilum and seedcoat, their average weights and their behaviour to modifications of soaking and cooking. The figures for hardness of the soybeans after soaking and cooking were obtained by measurements with the Penetrometer on 10–13 beans and the average figures are given in Table II. Values of 35–40 show the beans are soft but values below 20 show they are too hard.

Some green-coloured beans were among the 22 varieties: Ogden, Norchief, Hardome and Grant. Such beans are undesirable for making miso because the colour of the miso becomes dark and dirty, resulting in a loss of appeal to the consumer. Some varieties have the black or brown hilum surrounded by irregular black or brown areas (for example, Norchief, Shelby, Ford and Lindarin). These soybeans are undesirable, too, because they discolour the miso more strongly than those soybeans which have only a black hilum. Yellow-coloured seedcoat and hilum are most desirable.

In previous experiments, soybeans were found to absorb water very well after being soaked for 5 h at 25°C but the new crop soybeans did not absorb water well under these conditions. The difference lay in the original moisture content of the beans and its effect on water absorption. Beans with a low moisture content have a slower rate of water absorption than beans with a high moisture content. Details of these findings will be reported independently by Dr. A. K. Smith and Mr. A. M. Nash of the Northern Laboratory. As mentioned, beans of some varieties, like Harosoy and Comet, have a slower rate of water absorption and do not manifest uniform softening. Almost one-half of the beans of the Comet variety did not absorb water, even after soaking for 24 h at 25°C in water. Only beans which absorb water completely can be cooked thoroughly. Since all the varieties of soybeans had to be soaked in water for over 5 h, they were all soaked for 16–17 h at 25°C.

Table II. Characteristics of 22 varieties of U.S. soybean and their degree of hardness after varying soaking and cooking conditions in preparation for miso fermentation

Variety	Weight of 1,000 beans, g	Colour		Comment	Soaking and cooking conditions		
		Hilum	Seedcoat		1[a]	2[b]	3[c]
(1) Ogden	150·0	thin black	thin greenish-yellow	mixed with thin greenish-yellow beans	20·3	35·5	45·0
(2) Wabash	158·5	brown	light yellow	mixed with thin greenish-yellow beans	26·5	38·9	45·5
(3) Monroe	156·0	yellow	yellow		17·4	32·9	42·9
(4) Acme	139·0	yellow	yellow		34·0	38·4	43·7
(5) S 100	149·0	thin brown	yellow	plaits and cracks in seedcoat	17·7	25·2	36·0
(6) Capital	135·5	yellow	yellow		18·9	33·4	33·3
(7) Norchief	166·0	black	thin greenish-yellow	black hilum surrounded by irregular black area	28·2	41·0	41·2
(8) Flambeau	177·0	black	thin greenish-yellow	dirty colour, some have plaits and cracks in seedcoat	29·4	39·7	39·0
(9) Shelby	163·0	black	yellow	black hilum surrounded by irregular black area	24·4	35·3	42·9
(10) Mandarin	204·5	yellow	yellow		19·3	26·6	29·4
(11) Ford	167·5	black	yellow	black hilum surrounded by irregular black area	26·3	45·7	34·6
(12) Hardome	170·5	thin green	thin greenish-yellow		22·1	35·5	40·6

(13) Renville	183·0	brown	yellow		12·1	23·4	24·6
(14) Comet	136·0	yellow	yellow	plaits in seedcoat	20·3	29·6	35·6
(15) Lindarin	159·0	brown	yellow	some have brown hilum surrounded by irregular brown area	35·5	35·0	44·0
(16) Perry	173·5	brownish-black	yellow		25·0	25·1	32·8
(17) Blackhawk	172·5	brown	yellow	some are round	28·5	26·1	39·1
(18) Grant	160·0	black	thin greenish-yellow		18·6	24·4	33·8
(19) Jackson	147·0	thin brown	yellow	mixed with thin greenish-yellow beans	14·3	34·1	28·8
(20) Lee	123·0	grey	yellow	mixed with thin greenish-yellow beans	12·6	24·9	28·8
(21) Dortchsoy	98·5	yellow	thin brownish-yellow		9·2	19·7	24·9
(22) Dorman	147·5	thin brown	yellow		16·9	25·2	28·2

[a] Soybeans were soaked in water for 16 h at 25°C and then cooked for 1 h at 5 p.s.i. and allowed to stand for 1h.
[b] Soybeans were soaked in water for 16·5 h at 25°C and then cooked for 2 h at 5 p.s.i. and allowed to stand for 1 h.
[c] Soybeans were soaked in water for 17 h at 25°C and then cooked for 1 h at 10 p.s.i. and allowed to stand for 1 h.

None of the varieties of soybeans were completely cooked with 5 lb (p.s.i.) steam pressure for 1 h (followed by standing for 1 h). However, the Acme, Lindarin and Flambeau varieties were better cooked than the S 100, Mandarin, Renville and Grant varieties, which were still hard. At 5 p.s.i. for 2 h and additional standing for 1 h, a large number of the varieties of soybeans were thoroughly cooked—namely, Ogden, Wabash, Acme, Norchief, Flambeau, Shelby, Ford, Hardome and Lindarin—while S 100, Mandarin, Renville and Perry were still hard. Almost all varieties were fully cooked after treatment with 10 p.s.i. for 1 h and additional standing for 1 h. Ogden, Wabash, Acme and Lindarin were especially soft; Renville and Mandarin were still a little hard.

Mandarin has a yellow seedcoat and hilum, and is the largest in size of the 22 varieties. However, it was disappointing to find that it has a slower rate of water absorption and cannot easily be completely cooked.

Two varieties of U.S. soybean—Lee and Dortchsoy—are very small in size. Such small soybeans are more suitable for making natto than for making miso in Japan. Many of the Dortchsoy beans do not absorb water even after soaking for 24 h at 25°C, and as many as 40 per cent of the beans failed to absorb water.

As controls, eight different samples of Japanese soybeans and one kind of Chinese soybean were studied in the same fashion as were the 22 U.S. varieties reported in Table II. The results are shown in Table III. Five kinds of Japanese beans represent two seasons' crops; the year follows the variety name. The Japanese designations are not varieties but refer to the state in which they were harvested, except for Akita, which is a variety. All Japanese beans belonged to grade 2. The soaking and cooking conditions marked 1, 2 and 3 are the same as those described in Table II.

Weights of U.S. soybeans varied from 130 to 183 g per 1,000 beans, except Mandarin, which was 204 g. On the other hand, weights of all the Japanese soybeans exceeded 200 g, including one of 583 g (Nagano). Hokkaido-Akita is a representative soybean for miso-making in Japan. The miso manufacturers prefer large-sized soybeans and claim that they are more easily cooked and have a smaller amount of seedcoat.

None of the varieties of soybeans, whether U.S. or Japanese, were completely cooked with 5 p.s.i. for 1 h. However, there

Table III. Characteristics of 9 samples of Japanese soybean and their degree of hardness after varying soaking and cooking conditions in preparation for miso fermentation

Designation	Weight of 1,000 beans, g	Colour		Comment	Soaking and cooking conditions		
		Hilum	Seedcoat		1[a]	2[a]	3[a]
(1) Hokkaido-Akita (1957)	231·5	dark brown	yellow		26·9	51·1	52·1
(2) Iwate (1957)	211·5	yellow	yellow		26·3	47·7	52·1
(3) Miyagi (1957)	219·5	yellow, brown	yellow	mixture of a few varieties	29·3	43·6	51·1
(4) Nagano (1957)	583·0	dark brown	yellow	trade name Ginkaku	22·2	43·5	42·0
(5) Chinese (1957)	156·5	brown, yellow	yellow, greenish-yellow	mixture of a few varieties	25·3	36·2	47·1
(6) Hokkaido-Akita (1958)	270·5	dark brown	yellow		29·6	52·9	52·3
(7) Aomori (1958)	210·5	yellow, brown	yellow	mixture of a few varieties, dirty	25·2	49·2	49·7
(8) Miyagi (1958)	208·5	yellow, brown	yellow	mixture of a few varieties, foreign matter	34·0	49·9	46·4
(9) Nagano (1958)	253·5	brown	yellow		22·6	41·6	41·2

[a] Same conditions used as in Table II.

were marked differences in hardness between Japanese and U.S. soybeans as shown in the above tables. Furthermore, all the varieties of Japanese soybean were fully cooked with 5 p.s.i. for 2 h and with 10 p.s.i. for 1 h. Some were cooked in excess. However, subjected to the same pressures and cooking times, U.S. soybeans were not completely cooked.

Each lot of soybeans contained large and small beans. We were interested to learn whether there were differences of hardness between large- and small-sized beans in a single variety. Six varieties were studied and 10 g of beans of each variety were soaked in distilled water for 20 h at 24°C, cooked with steam at 5 p.s.i. for 1 h, and then allowed to stand for 1 h.

The hardness of the steam-cooked soybeans was again measured by a Penetrometer. The results are presented in Table IV.

After cooking, all varieties contained beans that were too hard or too soft. This was true irrespective of whether the variety had a large-sized bean or small-sized one. Values for beans that are too hard or too soft are enclosed in parentheses in Table IV. Many parentheses in some varieties indicate a highly variable hardness and thus the beans are not suitable for making miso and natto.

These results demonstrate that there is no marked difference in softness between large- and small-sized soybeans after cooking under the conditions listed above.

Ten grams of each of 6 varieties of U.S. soybeans were soaked in distilled water for a varying number of hours at about 25°C. They were then cooked by steam at varying pressures and lengths of time and allowed to stand for 1 h. As before, the hardness of steam-cooked soybeans was measured with a Penetrometer. The results are shown in Table V. The figures are averages for 10 to 13 beans. The numbers in parentheses indicate the number of beans which were either too hard or too soft for use in the miso fermentation and which were not incorporated in the average figures presented. If the number in parentheses after a variety is high, this indicates a highly variable hardness and that the beans are not suitable for miso or natto fermentation.

After soaking for 5 and 15·5 h in water, soybeans were not cooked completely with 5 p.s.i. for 1 h but were well cooked at 5 p.s.i. for 2 h. The dark colour of steam-cooked soybeans is not

Table IV. Comparison of hardness of soybeans of large and small size from the same variety

Variety	Weight of 1,000 beans, g	Size	Weight of ea. 1,000 beans, g	Hardness													Average
Chippewa	173·35	Small	100·0	36	20	34	18	21	20	15	22	21	15	(40)	20		22·0
		Large	217·5	(42)	18	29	20	21	26	17	17	21	28	31	23		22·8
Adams	144·15	Small	99·5	29	31	37	36	30	(17)	28	34	33	37	33	22		30·9
		Large	195·5	30	(38)	20	23	20	23	27	27	25	34	32	27		26·1
Lincoln	143·15	Small	94·0	38	32	32	33	18	18	18	30	26	15	36	19		26·2
		Large	211·5	25	21	21	(42)	(47)	22	30	32	20	19	(43)	28		24·2
Clark	160·0	Small	120·0	(33)	26	24	27	17	16	15	18	22	22	(12)	20		20·7
		Large	230·0	19	20	17	(28)	15	23	13	23	24	15	18	17		18·5
Harosoy	173·55	Small[a]	129·5	21	19	(41)	22	35	35	(16)	27	20	20	(13)	32		25·7
		Large	238·0	20	22	28	32	19	25	20	20	(40)	34	23	(45)		24·3
Hawkeye	166·0	Small	129·5	23	19	21	24	17	23	(33)	29	(45)	(32)	16	14		20·8
		Large	225·0	24	31	21	29	(35)	31	18	24	16	15	17	18		21·3

[a] Six beans in 36 beans (16·7 per cent) did not absorb water after 20 h.

Table V. Various soaking and cooking experiments with 6 varieties of soybean

Soaking time, h	Cooking time, lb	Cooking time, h	Weight of 1,000 beans: Seedcoat: Hilum:	Chippewa 173·30 yellow black	Harosoy 173·55 yellow white	Hawkeye 166·00 yellow black	Lincoln 143·15 yellow black	Clark 160·00 yellow black	Adams 144·15 yellow light brown
5	5	1		21·7 (1)	18·7 (2)	16·0 (2)	20·3 (1)	16·8 (2)	25·5 (0)
15·5	5	1		17·8 (4)	19·1 (3)	21·1 (2)	19·4 (4)	19·4 (2)	26·3 (4)
15·5	5	2		39·0 (2)	37·7 (2)	30·2 (0)	30·8 (1)	26·6 (2)	39·8 (0)
5	10	1		41·3 (2)	33·6 (1)	32·6 (2)	34·0 (1)	35·9 (1)	41·1 (2)
16	10	1		41·1 (0)	42·1 (0)	34·8 (0)	38·3 (0)	36·6 (2)	40·9 (0)
5	15	1		40·1 (1)	40·0 (3)	33·0 (1)	30·6 (1)	38·7 (1)	36·4 (1)
16	15	1		35·5 (0)	37·8 (3)	30·1 (3)	36·0 (0)	34·3 (1)	34·3 (0)

good for Shinshu-type white miso. As expected, cooking at a high pressure for a long period of time caused the soybeans to become dark brown. In making Shinshu miso, steam-cooking with little or no pressure is desired. Judging from the results obtained with the six varieties, Adams is a good soybean to use because it has a light yellow seedcoat and a thin yellow-brown hilum, is easily cooked, and is light in colour after cooking. The variety Clark cooked poorly.

All six varieties of soybeans were completely cooked using the soaking and cooking conditions of Table V. Adams and Chippewa were especially soft, while Clark and Hawkeye were slightly harder. A large autoclave for sterilization was used for experiments with 15 p.s.i. and hence the steam was dry. As a result, the cooked soybeans showed low values of hardness.

Discussion

Soybeans imported into Japan are mostly grade No. 2 soybeans, often referred to as Illinois No. 2. Processors of soybeans in Japan can be divided into two groups: (1) modern oil processors, and (2) the makers of traditional foods such as miso, natto and tofu. In the traditional food process, such as the manufacture of miso, whole beans must be soaked in cold water and cooked with steam. The time necessary for soaking varies according to the properties of the soybean, the kind of product desired and the temperature and hardness of the water. When Sendai miso, for example, is made in Japan, soybeans are soaked in water for 15–18 h with the water at a temperature of 12–15°C. Soybeans of Illinois No. 2 are a mixture of many varieties. Consequently, many Japanese processors have stated that U.S. soybeans have a slower and non-uniform rate of water absorption and cooking. To solve these problems, one must be acquainted with the properties of each variety in the mixture. Miso manufacturers prefer large beans of uniform size with a very light yellow colour and a white hilum. They wish to soften the soybeans by very rapid cooking. The standard size of Japanese beans ranges from small (1,000 beans weigh 150–250 g) to medium (250–350 g) to large (350–450 g). Manchurian beans, which were used in Japan before World War II, range in weight from small (110–140 g) to medium

(140–170 g) to large (170–200 g). From our data one can see that U.S. soybeans belong to the small-sized group. It has also been found that many varieties of U.S. soybeans absorb water at a slower rate and some beans do not absorb water at all. The beans which do not absorb water cannot be cooked. The most important fact is that if there are some uncooked hard beans among the cooked soft beans, miso cannot be made from them. Our data also show that Japanese soybeans cooked more easily than U.S. soybeans. It may be added that the smell of Japanese soybeans, while cooking, is different from that of U.S. soybeans, and there is also a difference in the taste after cooking.

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The Disintegration of Yeast with Dry Ice and Some Properties of the Extract

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Summary. A simple procedure for the disintegration of yeast cells, by which litre quantities of cell extract (approximately 11 per cent dry weight) may be obtained, is described. The fresh yeast cake is treated with dry ice in a high speed electric homogenizer after which it is left to thaw out at 0°. No addition of buffer has to be made, and the cell juice obtained after centrifugation of the resulting slurry appears to contain different subcellular particles and various enzymes in a relatively native state.

When the extract is incubated at higher temperatures, proteolysis will rapidly change the electrophoretic and enzymatic properties, which indicates that procedures involving autolysis may be dangerous when information about the chemical composition of the native enzymes is desired.

Introduction

Yeast is a favourite material for the preparation of subcellular particles, various enzymes and other compounds of interest to the biochemist and biologist. The classical autolysis procedure of Lebedew and other methods involving wet autolysis are widely used as preliminary steps for the preparation of enzymes.[1] Such procedures are, however, not suitable for the study of subcellular particles; proteolytic activity during the autolysis and extraction may also cause undesirable changes in the composition of the extract. Different methods have been described for the mechanical disintegration of yeast cells[2–4] but these require special equipment, and they are usually very time-consuming if large quantities of extract are desired. The disintegration procedure to be described has a relatively low disintegration effect, but this is often of secondary importance when such inexpensive cell material as commercial yeast is used. It appears to give an extract which corresponds relatively closely to the native cell cytoplasm, and it is easy to obtain litre quantities of such a cell juice. Rapid freezing alone will change the permeability properties of yeast,

and Krebs *et al.*[5] have utilized whole cells exposed to the temperature of dry ice for certain enzyme experiments. Repeated freezing and thawing will also cause partial lysis of some types of yeast, but complete disintegration of the cells is not usually obtained without mechanical treatment of the frozen cells.

Description of the Disintegration Technique

Small pieces of dry ice (solid CO_2) are first homogenized to a fine powder in an electric homogenizer.* The fresh yeast cake is then broken into pieces and run together with the dry ice for a suitable time. The shaping and sharpness of the metal knives have not been found to be of critical importance, and either stainless steel or heavy glass containers may be used. Depending on the size of the containers, 50–200 g of fresh yeast may be conveniently treated with an approximately equal volume of dry ice. When an extract from inexpensive commercial yeast is desired, 1–2 min homogenization time is usually adequate, since prolonged treatment of the cells will increase the efficiency relatively little. When large quantities of extract are desired, it is therefore easier to treat new batches of fresh cells and dry ice.

The homogenized material is left to thaw out in a beaker placed in running cold water or in an ice bath. The slurry obtained is then centrifuged at a suitable speed to remove remaining whole cells and large cell fragments. Baker's yeast (27 per cent dry weight) has been found to give approximately 20 ml of extract (10–12 per cent dry weight) per 100 g fresh yeast after centrifugation at 3,000*g* for 10 min. Brewer's yeast, which usually has a higher water content and is easier to disintegrate, may give almost twice this volume of extract.

Fig. 1 shows the appearance of baker's yeast homogenized with dry ice for 2 min. Only 10–20 per cent of the cells are completely emptied of their cytoplasmic content after such a short treatment, but a large fraction of the remaining cells are partially damaged. The treated cells may be washed with water or a suitable buffer to increase the total yield of extract, but this is not advisable when

* The MSE ATO-Mix Laboratory Blender and the Kenwood Electric Food Mixer Model A700 have been used by the authors for this purpose, but various other high speed homogenizers appear to be equally suitable.

the native cell juice is desired. Other methods are probably more useful when complete disintegration of small quantities of cells is desired, but cell wall fragments which are relatively free from cytoplasmic material may be prepared by repeated homogenizations and washings of the cell material.

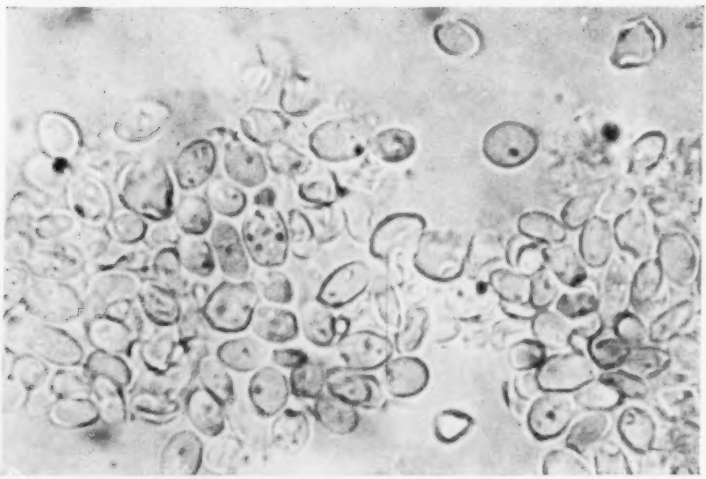

Fig. 1. Photomicrograph of baker's yeast disintegrated with dry ice in a high speed homogenizer

Some Properties of the Extract

Yeast contains primitive mitochondria, which may be stained with Janus Green B.[6] Nossal[7] has obtained granules containing a number of dehydrogenases from baker's yeast, and such particles are present in the extract obtained by the dry ice procedure. They may be purified by fractional centrifugation and washing with a suitable sucrose–versene–phosphate buffer and are stained pink in the presence of Janus Green B. Practically all the dehydrogenase activity as measured by the reduction of methylene blue or 2,6-dichlorophenol indophenol in the presence of succinate, fumarate or malate is associated with these particles, which are centrifuged down at 35,000*g* for 10 min.

The ribosomal particles in the extract were studied with the aid of the Spinco Analytical Ultracentrifuge Model E. Fig. 2 illustrates a sedimentation pattern, obtained after removal of the mitochondria, in which two distinct classes of particles are visible. The sedimentation coefficients obtained are relatively low owing to the high viscosity of the extract. Centrifugations of serial dilutions of the extract gave values which, when extrapolated to zero concentration, show that the largest and more rapidly

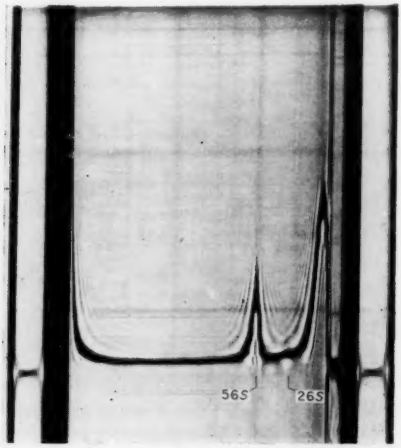

Fig. 2. Ultracentrifugal schlieren diagram of an extract (approximately 10 per cent dry weight) of baker's yeast photographed after 19 min at 29,500 rev/min. The numbers on the photograph give the uncorrected sedimentation coefficients in Svedberg units

sedimenting particle fraction corresponds to the 80 S component observed by Chao and Schachman[8] and Bowen *et al.*[9] The presence of 0·02M Mg^{2+} in the extract was not found to change the sedimentation patterns obtained appreciably.

Undisrupted baker's yeast has a very low rate of endogenous fermentation, and the addition of glucose to the cells does not increase this rate appreciably. A cell-free extract ferments glucose at a high rate, but it is difficult to estimate the amount of CO_2 produced per mole glucose added because of the presence of

large amounts of endogenous substrates. The enzyme enolase can be detected in the crude extract by the optical method of Warburg and Christian.[10] Interfering side reactions in the extract probably make this method unsuitable for reliable determinations of absolute amounts of the enzyme. It is, however, of some interest that the enolase activity per milligram of protein was found to be three times higher in extracts from anaerobically grown brewer's yeast than in extracts from baker's yeast grown with strong aeration.

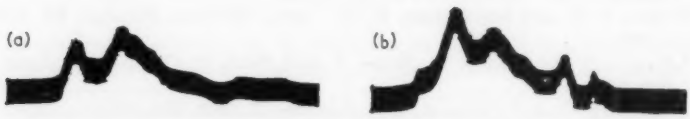

Fig. 3. Electrophoretic boundary patterns obtained with particle-free extracts of baker's yeast. LKB Moving Boundary Apparatus with a phosphate–borate buffer pH 8·2; $\mu = 0{\cdot}05$. The photographs show the descending side for negatively charged compounds after 42 min at 13 mA. (a) Extract maintained at 0°; (b) extract incubated at 37° for 7 h in the presence of traces of penicillin and streptomycin to prevent bacterial growth. Both extracts were dialysed for 12 h at 0° against the buffer used in the electrophoresis experiment

Yeast contains at least two enzymes with proteolytic activity,[11] and it is obvious that these may have undesirable effects if a cell extract is kept under conditions which favour proteolysis. As shown in Fig. 3, the electrophoretic pattern of the particle-free extract is thus markedly altered during incubation at 37°. A further indication of proteolysis under such conditions is that the pH of an extract left overnight at 37° will drop from 5·5 to 4·5 and that the total amount of material precipitable with trichloroacetic acid or acetone is drastically reduced. Various enzymes appear to be inactivated at different rates by the proteolytic enzymes. The dehydrogenase activities are particularly sensitive, and they are reduced to about 50 per cent in 24 h even if the extract is kept in an ice bath. The enolase activity is much more stable under similar conditions, which is interesting in view of the data of Nylander and Malmström,[12] which show that this enzyme can be extensively degraded with peptidases without loss of activity.

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Bacterial Sulphide Production from Sulphate-Enriched Spent Distillery Liquor—II

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Summary. Accumulation of volatile acids produced during anaerobic fermentation of distillery-spent liquor decreased the activity of *Desulphovibrio desulphuricans* employed in previously communicated studies; this caused a decrease in the reduction of sulphates to sulphides. An enriched strain of *Desulphovibrio rubentschikii* made in a synthetic mineral medium (NH_4Cl, 1·0 g; $MgSO_4 \cdot 7H_2O$, 2·0 g; NaCl, 10·0 g; $CaSO_4$, 1·0 g; calcium acetate, 1·0 g; K_2HPO_4, 0·5 g; Mohrs' salt, trace in 1 l.) using calcium acetate as the substrate and carbon from the tap-water was gradually built up to a 3-l. volume. The amount of hydrogen sulphide in this medium was found to be 225 mg/l. The fermentation was next carried out in a synthetic medium containing volatile acids from distillery-spent liquor as the only substrate. A 3-l. fermentation set up with diluted distillery-spent liquor (300 ml of raw liquor in 3,000 ml of water), neutralized with NaOH to pH 7·2 and supplemented with calcium sulphate and 0·1 per cent urea, showed sulphide productions (calculated as milligrams of hydrogen sulphide per litre of raw distillery-spent liquid) of 2,000, 4,000 and 6,000 with 0·1, 1·0 and 2·0 per cent calcium sulphate in the charge respectively. The volatile acid content, expressed as acetic acid, was 18,564 mg/litre of raw distillery-spent liquor on an average throughout the study. The ratio of hydrogen sulphide produced per litre of raw distillery-spent liquor to the amount of volatile acids developed expressed in milliequivalents per litre of raw liquor during fermentation increased gradually from 0·1367 to 0·5567 in the presence of 0·1 to 2·0 per cent of calcium sulphate in the charge.

Preface

In a previous communication (Part I, in press), studies carried out with organisms of the type *Desulphovibrio desulphuricans* in the bacterial reduction of sulphates to sulphides using distillery-spent liquor as carbon and hydrogen donor were reported. It was pointed out that increased yields of sulphide might be obtained by employing in the microbial sulphate-reduction a volatile-acid-utilizing

bacteria of the type *Desulphovibrio rubentschickii*. Studies subsequently conducted on the enrichment, isolation and fermentation characteristics of a specific volatile-acid-utilizing strain of sulphate reducers isolated from Calcutta City Sewage are reported. A comparative study on the sulphide fermentation with the agency of both *D. desulphuricans* and *D. rubentschickii* using distillery-spent liquor as hydrogen-donor has also been made. The influence of volatile acids on the bacterial reduction of sulphates to sulphides is also reported here.

Introduction

In Russia, investigations have been carried out on the enhancement of sulphur production in natural waters as reported by Murzaev (1950). Butlin *et al.* (1954, 1956) and Burgess *et al.* (1958) demonstrated the possibilities of bacterial sulphate reduction using sewage sludge as a cheap source of carbon and hydrogen donors. A comparable process using yeast plant effluent in a similar sulphide fermentation has recently been developed in Czechoslovakia. The process also resulted in a considerable reduction in the 5-day B.O.D. of the effluent from 6800 ppm to 1200 ppm as reported by Barta and Greģr (1956). At a symposium on the microbial production of sulphides held at Teddington, England, Postgate (1954), Butlin and Thomas (1954) and Senez (1954) discussed the theoretical considerations and the raw materials necessary for autotrophic reduction of sulphates. By a batch process using raw sewage sludge (5 per cent solids) supplemented with gypsum, a yield of 1·1 per cent of sulphide (based on the weight of sludge) in a 4-week period has been reported from the Chemical Research Laboratory, Teddington, England. The occurrence of highly active sulphate-reducing strains is also reported by Miller (1949, 1950). He obtained in synthetic mineral media, 2000–2500 mg of hydrogen sulphide per litre, and 3586 mg, 3346 mg and 3339 mg of sulphides of zinc, lead and cadmium per litre of substrate respectively.

Recent investigations on the use of distillery-spent liquor (Alcoholic Distillation Slop) in the production of methane reveal the possibility of employing the carbon and hydrogen of this waste in other microbial processes. Hideo Ono *et al.* (1958a) described

the use of the slop in methane fermentations on a continuous basis. They also reported about 90 per cent reduction in the B.O.D. level by the activated sludge process of the methane-fermented digestive fluid. Hideo Ono *et al.* (1958c) further reported the production of about 120 mμg per ml of Vitamin B_{12} during a methane fermentation employing alcoholic distillation slop. The proportion of B_{12} recovered by the activated sludge process proved to be satisfactory and the process has scope for use on an industrial scale. Hideo Ono *et al.* (1958b) described the production of a gas containing 51 per cent CO_2, 44 per cent CH_4, 3 per cent H_2, and 150 mμg of Vitamin B_{12} per ml of digested fluid during thermophilic (51°–53°C) digestion of alcoholic distillation slop. The process also resulted in a B.O.D. reduction of 75–80 per cent. Ghose *et al.* (1960) reported on the high rate of fermentation of distillery waste by enriched methane cultures and showed that there are practically no effects due to initial pH, dilution and supplementation of $CaCO_3$ on the reduction of C.O.D. values of raw waste. In six days, the reduction in the C.O.D. value of the raw waste was between 61 and 65 per cent.

The present studies have been undertaken with a view to utilizing distillery-spent liquor in the bacterial reduction of sulphates to sulphides.

Materials and Methods

A. Enrichment of Sulphate-Reducing Bacteria Employed in the Studies

The general procedure used for the isolation and enrichment of the two sulphate-reducing organisms (*D. desulphuricans* and *D. rubentschickii*) was derived from the method outlined by Starkey (1938). The source of *D. desulphuricans* was, as previously, the discharge of a semi-continuous methane fermentation run in the laboratory in a synthetic mineral medium employing ethyl alcohol as the substrate and reported earlier by Ghose *et al.* (1959). The basal medium contained (per litre): $CaCl_2 \cdot 6H_2O$, 0·15 g; NH_4Cl, 1·0 g; $MgSO_4 \cdot 7H_2O$, 2·0 g; $Na_2SO_4 \cdot 10H_2O$, 4–7 g; ethyl alcohol (absolute), 3·5–6 g; K_2HPO_4, 0·5 g and a trace of Mohr's salt. The volatile-acid-utilizing sulphate reducers were isolated from Calcutta City Sewage and enriched in the basal medium

containing (per litre): K_2HPO_4, 0·5 g; NH_4Cl, 1·0 g; $CaSO_4$, 1·0 g; $MgSO_4 \cdot 7H_2O$, 2 g; NaCl, 10·0 g and $(CH_3COO)_2Ca$, 1·00 g. The media were previously boiled and cooled under a N_2-gas stream to stop re-absorption of oxygen from the atmosphere. A trace of Mohr's salt was also used as before and the media incubated at 37°C. Culture tubes were sealed under alkaline pyrogallol, plugged, and incubated anaerobically according to Ghose (1955). Distilled water and reagent grade chemicals were used in the preparation of media in all cases.

Transfers from one to the next series of sub-cultures were conducted every 72 h of incubation at 37°C. Indications for transfer were either: (1) complete darkening of the walls of the culture vessels (tubes and bottles) due to deposition of lustrous Fe_2S_3; or (2) a reduction of about 70 per cent in the acetic acid initially present. Particular care was taken to pipette the bed material from the tubes or bottles, where perfect anaerobiosis existed, for its use as inoculum into the next series. The bacteria appeared under the microscope as highly motile, short rods, obligatory anaerobic Gram-negative vibrio cells with an optimum pH for growth and proliferation in about the neutral range. The culture was maintained in liquid medium throughout the study and two transfers made every week.

B. Analytical Methods

Iodometric estimation of the sulphide in fermented liquors as used by Starkey (1938) has been followed in these studies. Estimation of sulphide in the gas phase was made by the passage of the gas produced during fermentation through alkaline cadmium hydroxide solution prepared by dissolving 4·3 g of cadmium sulphate ($3CdSO_4 \cdot 8H_2O$) in water, adding 0·3 g of sodium hydroxide dissolved in water and finally making up the volume to 1 l., thus trapping all the H_2S quantitatively as yellow CdS, as proposed by Morris *et al.* (1957). The amount of sulphate in the raw and fermented samples was determined by the gravimetric method of Treadwell (1930) and Kolthoff and Sandell (1947), as used by Ghose (1955) in a modified procedure in which barium sulphate is precipitated with 5 per cent barium chloride solution. The apparatus used for the separation of fatty acids from the raw and fermented samples by steam-distillation and the estimation of the

fatty acids were the same as those used by Ghose (1955) for the estimation of various lower fatty acids produced in bacterial fermentations. Various volumes of raw and fermented liquors, after different dilutions, were steam distilled and 20-ml fractions were titrated with 0·01 N NaOH. Standard curves representing the end-points of the steam distillation of the volatile acids in samples of distillery-spent liquor are shown in Fig.1. Values for chemical

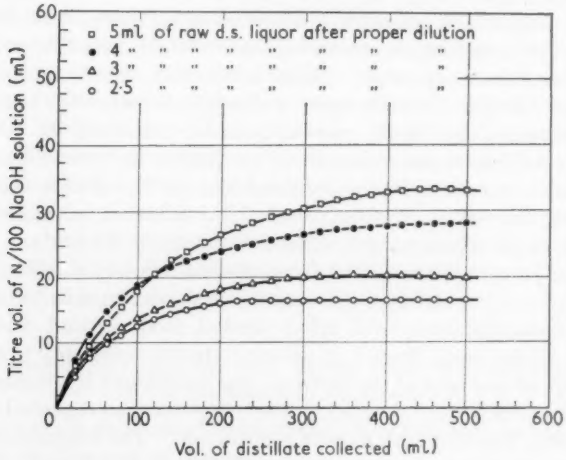

Fig. 1. Steam distillation of distillery-spent liquor for complete removal of the volatile acids

oxygen demand (C.O.D.) of raw and fermented samples were estimated by the standard dichromate method (*Standard Methods for the Examination of Water, Sewage and Industrial Wastes*, American Public Health Association, Inc., 1955, p. 333).

Experiments and Results

A. Influence of Volatile Acids in the Bacterial Sulphate Reduction

It has been reported in an earlier communication by Ghose and Basu (Part I, in press) how the accumulation of steam-volatile acids in the fermenting medium impedes the yield of sulphide using

ethyl alcohol as the carbon source in cultures of *D. desulphuricans*. It was illustrated that a gradual decrease in the ratio of sulphide produced (mmoles/litre) to volatile acids generated (meq/litre) during fermentation took place. Over a period of 10 days this ratio fell from 0·116 to 0·052.

Rubentschik (1928) demonstrated for the first time that lower fatty acids formed by microbial decomposition of cellulose are utilized by anaerobic sulphate reducers as H-donors in the reduction of sulphate to sulphide. Later, Baars (1930) distinguished between two species, *D. desulphuricans* and *D. rubentschickii*, on the basis of their capacity to utilize fatty acids as hydrogen donors and, accordingly, the only species of sulphate reducers capable of metabolizing fatty acids was named *D. rubentschickii*. Ghose (1955) established the existence of an inhibitory action by lower fatty acids on the reduction of sulphates by the Zürich strain of *D. desulphuricans*. The degree of this inhibition increased with increase in the chain length of the acid up to the C_4-acid.

In the present studies, a 3-l. fermentation using synthetic acetate medium and *D. rubentschickii* was set up similar to that conducted in *D. desulphuricans* with ethyl alcohol medium and described earlier (Ghose *et al.*, Part I, in press). On the tenth day following start-up, 50 per cent of the medium was discharged by decantation and 1·5 l. was freshly charged. The operation was repeated on the 17th, 24th, 31st and 37th days of incubation. The fermentors were discharged under CO_2-gas pressure. These fermented samples showed considerable reduction in their initial volatile acids (from 1,008 mg to only 100 mg of volatile acids expressed as acetic acid per litre). Owing to the feeble growth at the initial adaptation phase of the acid-utilizing sulphate reducers and to permit undisturbed growth at the bottom of the fermentor, samples were withdrawn by decantation. The concentration of sulphide in the decanted samples of the fermented medium was in most cases around 95 mg of H_2S per litre, while the feed contained on an average 1,440 mg of total sulphate per litre. On the 45th day, 1·5 l. of the homogenous fermented medium was discharged and 1·5 l. of fresh medium was charged. This operation was repeated on the 54th, 59th, 63rd, 70th, 85th and 93rd days. The total sulphide produced in the liquid and gaseous phases was on an average 9·5 mmoles per litre of the medium as estimated at the

end of each cycle. This accounted for an overall reduction of total sulphate introduced into the system of 63·5 per cent. The steam-volatile acids remained nearly constant around 9·95 meq per litre of medium (597 mg of acetic acid per litre) corresponding to the initial acid concentration of 1,008 mg/litre. The difference between the volatile acid concentrations in this and the earlier stage of the fermentation is due to the heterogeneous system of the cultures. The ratio of mmoles of total sulphide produced per litre of the medium to total milliequivalents of volatile acids left per litre of the fermented medium was on an average 0·955. Thus, this ratio is about nine times higher than that obtained with a synthetic mineral medium using ethyl alcohol as H-donor in cultures of *D. desulphuricans*. Further, the above figure shows a decreasing trend in cultures of *D. desulphuricans* and an increase in the population of *D. rubentschickii*. It is evident, therefore, that while the accumulated volatile acids in cultures of *D. desulphuricans* inhibit sulphate reduction in synthetic medium, enriched cultures of *D. rubentschickii* rapidly utilize acetic acid in the bacterial reduction of sulphates to sulphides.

B. Utilization of Distillery-Spent Liquor in the Bacterial Reduction of Sulphates to Sulphides

The average analysis of the distillery-spent liquor used throughout the studies was as follows:

pH of the raw liquor, 3–4
Sp. gr. of the raw liquor, 1·4–1·5
Total solids of the raw liquor, 4–5 per cent
Total volatile matters, 65–70 per cent (dry basis)
Total sulphates (acid-soluble), 17–18 mmoles/litre
Total sulphides, nil
Chemical Oxygen Demand, 1,55,000–1,62,000 ppm
Total steam-volatile acids, 66·5–83·5 meq/litre

In the earlier studies by Ghose *et al.* (Part I, in press) it was reported that supplementation of urea in the medium to the extent of 0·01 per cent of the dilute (1:10) distillery-spent liquor in the sulphide fermentation increases the yield of sulphide. The beneficial effect of urea in the methane fermentation of spent distillery waste has been demonstrated by Ghose *et al.* (1960).

The percentage yields of sulphide based on the weight of distillery-spent liquor in various dilutions were 0·137 in 1:5 diluted d.s. liquor, 0·199 in 1:10 diluted d.s. liquor, and 0·221 in 1:20 diluted d.s. liquor, without any addition of urea, while corresponding figures in 1:5, 1:10 and 1:20 diluted d.s. liquor media were 0·205, 0·292 and 0·45 with supplementation with 0·1 per cent urea on raw d.s. liquor. The effect of urea on the yield of sulphide appeared to be feeble with increasing concentration of the distillery-spent liquor in the media. This N-source seems to have no pronounced

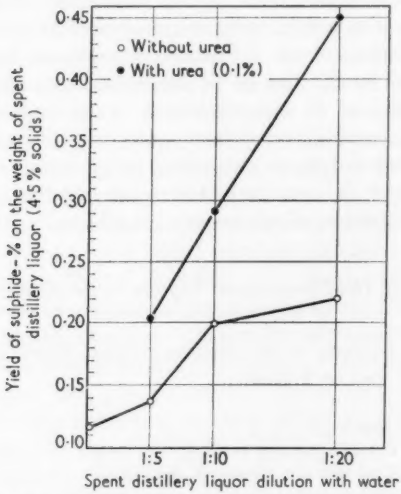

Fig. 2. Effect of urea on the sulphide fermentation in distillery-spent liquor

effect on the sulphide fermentation of raw liquor—the yield of sulphide being 0·116 per cent on the basis of the weight of the spent distillery liquor. Effects of urea on the sulphide fermentation in spent distillery liquor with various dilutions are shown in Fig. 2.

Studies on sulphide fermentation (batch) with diluted spent distillery liquor in D. desulphuricans *and* D. rubentschickii. Basal medium was prepared by diluting raw distillery-spent liquor (1:10) with

tap-water which was supplemented with 0·1 per cent urea, and the pH was adjusted to 7·1. Two 3-l. fermentors were set-up with this medium. Calcium sulphate, $CaSO_4 \cdot \frac{1}{2}H_2O$, (60g) was added to each. One of the fermentors was inoculated with 300 ml of a 3-day-old enriched culture of the strain *D. desulphuricans* (*I*) and the other with an equal volume of a similarly aged culture of *D. rubentschickii* (*II*); 50 ml of fermented medium were removed from the fermentors every 6 h and were analysed for pH, volatile acids, and sulphides. The total gas produced during these intervals was also measured. The results are tabulated in Tables I and II.

From these results it is clear that during anaerobic dissimilation of distillery-spent liquor the volatile acids which are generated in the process increase steadily in cultures of *D. desulphuricans* till about 36 meq/litre. The corresponding yield of

Table I. Batch fermentation of ten times diluted distillery-spent liquor supplemented with calcium sulphate, $CaSO_4 \cdot \frac{1}{2}H_2O$ (2 per cent) and urea (0·1 per cent) employing *D. desulphuricans*

Period of fermentation, h	Volume of gas collected (cumulative), ml	mg sulphide in gas/litre fermenting medium (cumulative)	mg sulphide/litre fermenting liquor in discharge	Milliequivalent volatile acids/litre fermenting medium	pH of fermenting medium
0	0	0	51	—	7·1
6	0	0	72	14·72	7·05
12	180	0	102	16·20	7·00
18	235	0	102	17·65	6·90
24	245	trace	110·5	19·20	,,
30	315	,,	126·5	22·20	,,
36	440	,,	126·6	22·30	,,
42	590	,,	153·0	23·60	,,
48	660	,,	170·0	24·20	,,
54	690	,,	170·0	24·20	,,
60	700	,,	174·0	24·40	,,
96	720	,,	356·0	26·60	,,
120	720	,,	340·0	35·40	,,
144[a]	720	201·1	306·0	38·40	,,
240	720	nil	302·0	38·40	,,
264	720	nil	302·0	38·40	,,

[a] At this stage the gas bubbler containing alkaline cadmium hydroxide was replaced by a new one and the content of yellow CdS was estimated from the absorption solution removed.

Table II. Batch fermentation of ten times diluted distillery-spent liquor supplemented with calcium sulphate, $CaSO_4 \cdot \frac{1}{2}H_2O$ (2 per cent) and urea (0·1 per cent) employing *D. rubentschickii*

Period of fermentation, h	Volume of gas collected (cumulative), ml	mg sulphide in gas/litre fermenting medium (cumulative)	mg sulphide/litre fermenting liquor in discharge	Milliequivalent volatile acids/litre fermenting medium	pH of fermenting medium
0	0	0	85·0	—	7·1
6	30	—	93·5	14·8	7·00
12	270	—	123·0	14·75	7·00
18	670	trace	127·5	13·25	7·00
24	1,135	,,	144·5	11·80	7·00
30	1,760	,,	148·5	10·30	6·90
36	2,050	,,	157·0	10·30	,,
42	2,305	,,	195·0	10·00	,,
48	2,945	,,	212·2	9·45	,,
54	3,345	,,	221·0	8·85	,,
60	3,700	,,	228·0	7·38	,,
96	4,000	,,	380·0	7·38	,,
120	4,150	,,	400·0	7·35	,,
144[a]	4,200	101·9	412·5	6·80	,,
240	4,250	nil	425·0	6·80	,,
264	4,290	nil	425·0	7·00	,,

[a] At this stage the gas bubbler containing alkaline cadmium hydroxide was replaced by a new one and the content of yellow CdS was estimated from the absorption solution removed.

sulphide is about 350 mg/litre. Further increase of volatile acids in the medium exercises a marked inhibitory effect on the yield of sulphide and it suppresses sulphate reduction to a level of about 300 mg/litre. Thus, the bacterial flora cannot tolerate accumulated volatile acids which have no possibility of elimination from the system. Contrary to this, the cultures of *D. rubentschickii* are capable of utilizing the volatile acids generated in the process, the concentrations of which therefore gradually decrease, and the yield of sulphide increases. Finally, the acid level falls to 6·8 meq/litre and thereafter becomes constant, the corresponding yield of sulphide being 425 mg/litre. Thus the volatile acids, after a steady increase from 14·8 meq/litre, become constant at 38·4 meq in *D. desulphuricans* while the cultures of *D. rubentschickii* reduce the initial value to 6·8 meq/litre. These are illustrated in Fig. 3.

The results show further that the production of gas by the

acid-utilizing cultures of *D. rubentschickii* (4,290 ml) is largely in excess of that by the culture of *D. desulphuricans* (720 ml) during the 264 h of anaerobic dissimilation. The yield of sulphide in the gas phase is, however, higher in the case of *D. desulphuricans* (201·1 mg/litre of fermenting medium) after a period of 144 h of fermentation than in the case of *D. rubentschickii* (101·9 mg/litre of fermenting medium) during the same period. The pH remained steady and constant at 6·9 in both cases, and no adjustment of pH was found necessary during the fermentations. The ratio of

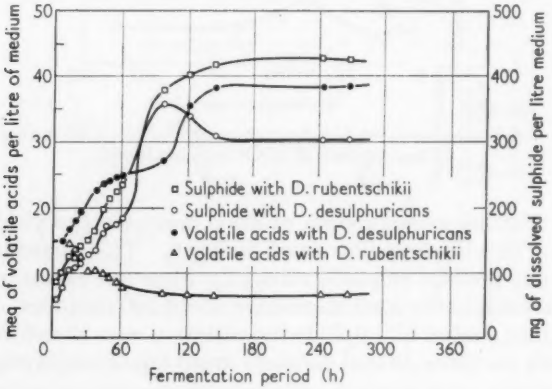

Fig. 3. Concentrations of volatile acids and sulphide per litre of medium during the batch fermentation of diluted distillery-spent liquor

mmoles of sulphide produced to the mequivalents of volatile acids increases with *D. rubentschickii* and decreases with *D. desulphuricans*. This is illustrated in Fig. 4.

Study on the semicontinuous sulphide fermentation with distillery-spent liquid employing D. desulphuricans *and* D. rubentschickii.

It has already been reported in the previous communication (Part I) how the accumulation of volatile acids inhibits the reduction of sulphate to sulphide by *D. desulphuricans* while fermenting raw distillery-spent liquor. The yield of sulphide increases gradually with increase in volatile acids up to about 175 meq of volatile acids per litre of fermenting medium, the corresponding figure of sulphide output being in the neighbourhood of 52 mmoles/

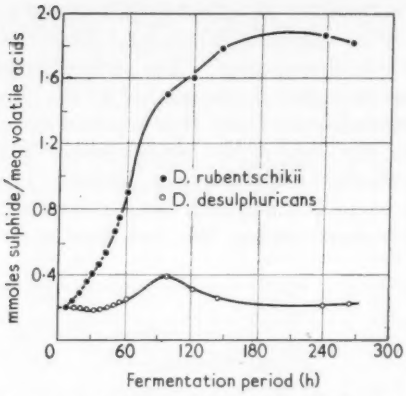

Fig. 4. Relation between sulphide production and volatile acids during the batch fermentation of distillery-spent liquor

litre. Further accumulation of acids decreases the yield of sulphide. This has been illustrated in Fig. 5. This particular strain could not produce sulphide efficiently when the concentration of volatile acids in the medium reached about 200 meq/litre.

Further studies on sulphide fermentation were therefore carried out with ten times diluted distillery-spent liquor employing strains

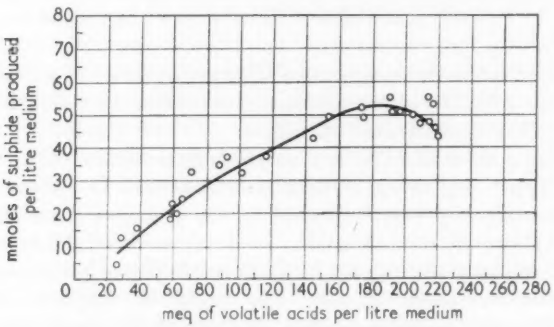

Fig. 5. Relation between sulphide yield and concentration of volatile acids during the semi-continuous sulphide fermentation in distillery-spent liquor with *D. desulphuricans*

of the type *D. rubentschickii* in a 3-l. glass fermentor. The medium contained 300 ml of raw distillery-spent liquor diluted with tap-water to a volume of 3 l. It was supplemented with 3 g of urea and 3 g of calcium sulphate ($CaSO_4 \cdot \frac{1}{2}H_2O$), and the pH adjusted to 7·2. A 3-day-old culture of *D. rubentschickii* was used as inoculum (10 per cent of medium). Samples (totalling 300 ml) showed a gradual increase of sulphide in the fermenting medium. On the 13th day following start-up, 1·5 l. of the fermented medium was replaced by concurrent feeding with 1·5 l. of fresh

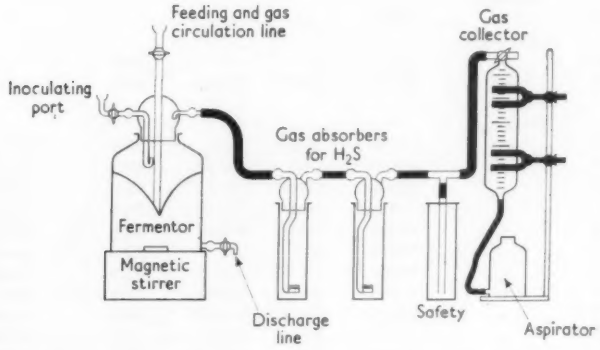

Fig. 6. Set-up for semi-continuous sulphide fermentation in distillery-spent liquor

medium (150 ml of raw spent liquor diluted to 1·5 l.) supplemented with 1 per cent $CaSO_4 \cdot \frac{1}{2}H_2O$ and 0·1 per cent urea. A sketch of the set-up used in the study is shown in Fig. 6.

The operation of feeding and discharge cycles was repeated on the 19th, 22nd and 30th days. The amount of supplemented $CaSO_4 \cdot \frac{1}{2}H_2O$ was next increased to 2 per cent in the charge. On the 36th day, 1·5 l. of the fermenting medium was similarly replaced by 1·5 l. of freshly prepared medium as above with the supplements of 2 per cent $CaSO_4 \cdot \frac{1}{2}H_2O$ and 0·1 per cent urea. These cyclic operations were repeated on the 44th, 50th, 57th, 64th, 76th, 83rd, 91st, and 98th days. The results are summarized in Table III.

Table III. Semi-continuous sulphide fermentation with supplemented and diluted distillery-spent liquor employing *D. rubentschickii*

Period of fermentation, days	Volume of charge and concurrent discharge, ml	mg sulphide/litre fermenting medium		Total	meq volatile acids/litre fermenting medium	Ratio of mmoles total sulphide produced to meq of volatile acids in the medium
		in medium discharged	in gas			
0	3,000 charged	—	—	—	—	—
2	100 discharged	52·6	—	—	—	—
8	,,	67·0	—	—	30·9	—
13	1,500 charged and discharged	161·5	18·35	179·85	32·0	0·165
19	,,	187·0	8·5	195·5	35·0	0·165
22	,,	170·0	8·5	178·5	33·3	0·158
30	,,	195·5	31·8	227·3	32·6	0·202
36	,,	324·0	58·4	360·4	30·8	0·344
44	,,	332·0	51·0	383·0	31·4	0·351
50	,,	399·0	55·1	454·1	29·8	0·448
57	,,	416·0	49·75	465·75	30·9	0·445
64	,,	416·0	46·75	462·75	28·7	0·472
76	,,	450·0	76·25	526·25	30·9	0·501
83	,,	527·0	70·8	597·8	27·8	0·633
91	,,	476·0	73·6	549·6	27·7	0·585
98	,,	501·0	72·4	573·4	—	—
105	,,	510·0	—	—	—	—

While fermenting distillery-spent liquor employing *D. desulphuricans* both the volatile acids and the sulphide produced increase up to a concentration of nearly 200 meq of volatile acids per litre of the medium—the yield of sulphide decreases with further increase in volatile acids (*vide* Fig. 5). The maximal yield of sulphide is 0·12 per cent on the weight of distillery-spent liquor (total solids 4·5 per cent). In the case of a fermentation employing *D. rubentschickii*, however, the volatile acids decrease steadily with time and there is a gradual increase in the level of sulphide yield. This seemingly illustrates the ability of this new strain to utilize the volatile acids of the distillery-spent liquor as a source of carbon

and hydrogen donors. The yield of sulphide is 0·415 per cent on the weight of the distillery-spent liquor.

Fig. 7 represents the ratio of mmoles of sulphide produced to mequivalents of volatile acids in the medium against the period of fermentation in days. This ratio also increases continuously showing utilization of the volatile acids of distillery-spent liquor by the acid-utilizing and sulphate-reducing flora cultivated in the system. In the case of fermentations with *D. desulphuricans*, the said ratio shows an initial phase of fall in the sulphide produced in relation to volatile acids used up which then reverses and rises up

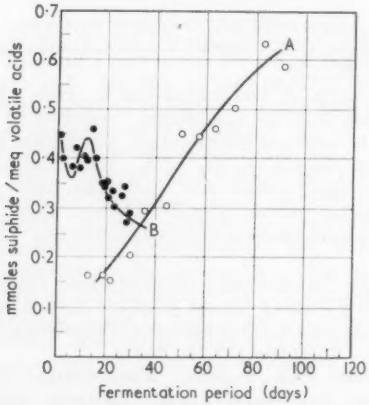

Fig. 7. Comparative yields of sulphide and volatile acids during semi-continuous sulphide fermentation in distillery-spent liquor with *D. rubentschickii* (A) and *D. desulphuricans* (B)

to a level of 0·4 and decreases again in the final phase. The initial fall may be explained as the lag phase assigned to the adaptation of the cultures to the new environment during replacement of the synthetic mineral medium (ethyl alcohol as substrate) by raw spent distillery liquor. The organisms having been adapted to the new substrate, sulphide fermentation resumes and hence the ratio increases. The final decrease in the ratio establishes the inhibitory action of the volatile acids on the sulphate reduction in spent liquor. The unused acids therefore accumulate in the medium and the yield of sulphide decreases progressively.

Occasional estimation of the Chemical Oxygen Demand values of the charges and discharges of the fermentors showed a decrease of about 78·3 per cent in both cases.

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Die technische Herstellung von klinischem Dextran durch gelenkte Synthese in wachsender Kultur

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Summary. A process is described for the production of a specific-molecular-weight dextran, e.g. clinical dextran, by using a modified form of controlled synthesis. The synthesis is carried out directly in a medium containing appropriate amounts of sucrose and low-molecular-weight dextran after inoculation with a culture of actively growing bacteria. The importance of an inoculum substantially freed from substances affecting the synthesis of dextran is demonstrated. The technique appears to offer a decided advantage over other well-known procedures. Technical details of the process and equipment are given.

Einleitung

Die technische Herstellung von niedermolekularem, i.b. klinischem Dextran erfolgt allgemein durch Hydrolyse hochmolekularen, nativen Dextrans, wie es in Kulturlösungen von dextranbildenden Bakterien anfällt. In der Notwendigkeit, das hochmolekulare Dextran partiell depolymerisieren zu müssen, liegen sowohl die Schwierigkeiten als auch die wesentlichen Ausbeuteverluste des hydrolytischen Verfahrens. Die Spaltung der Kette erfolgt willkürlich, zufällig. Das Hydrolyseprodukt ist sehr uneinheitlich. Es muß durch Fraktionieren von höher- und niedermolekularen Anteilen getrennt werden.

Es hat nicht an Versuchen gefehlt, die verlustreiche Depolymerisation zu umgehen und die Dextransynthese so zu lenken, daß im

wesentlichen nur Dextrane von einem bestimmten Molekulargewichtsbereich gebildet werden. In vorliegender Arbeit wird ein Verfahren beschrieben, das die Kontrolle und Lenkung der Synthese mit einfachen Maßnahmen gestattet und Dextrane von bestimmten Molekulargewichten wirtschaftlich herzustellen erlaubt.

Prinzip der gelenkten Synthese

Die Synthese des Dextrans wird so verstanden, daß der Glukosyl-Anteil der Saccharose durch ein Enzym, die Dextransucrase, auf einen geeigneten Akzeptor übertragen wird. Die Zahl dieser Transfer-Reaktionen bzw. die Zahl der verfügbaren Glukosyle bestimmt in Verbindung mit der Zahl der Akzeptoren den Polymerisationsgrad des entstehenden Dextrans. Durch die Variation des Verhältnisses Zahl der Glukosyle zu Zahl und Polymerisationsgrad der Akzeptor-Moleküle läßt sich ein bestimmtes Molekulargewicht einstellen. Dieses Verfahren wird als 'gelenkte Synthese' bezeichnet.

Als Glukosyl-Donator ist nur Saccharose geeignet. Als Glukosyl-Akzeptoren eignen sich besonders Dextrane unterschiedlichen Molekulargewichts, wobei nach Patat[1] dem hochmolekularen Dextran eine stärkere Glukosyl-Affinität zuzuschreiben ist. Neben Dextran ist eine Reihe einfacher Zucker in der Lage, die durch die Dextransucrase übertragbaren Glukosyle aufzunehmen.

Von Koepsell, Tsuchiya und Hellman[2–4] wurden die qualitativen und quantitativen Zusammenhänge der Akzeptor-Spezifität untersucht. Sie fanden einen prinzipiellen Unterschied zwischen der Akzeptor-Wirkung der Dextrane und der der niederen Zucker. Als Ergebnis der Polymerisation in Gegenwart niederer Zucker wurde ein bimodal verteiltes Dextran erhalten. Neben einem Anteil von sehr hohem Molekulargewicht entstanden niedermolekulare Dextrane und Oligosaccharide. Als Ergebnis der Polymerisation in Gegenwart von Dextran als Akzeptor wurde ein Dextran erhalten, welches dem Verhältnis Akzeptor-Konzentration und -Molekulargewicht zu Zahl der umgesetzten Glukosyle entsprach. Das bedeutet, daß die Affinität der niederen Zucker zum Glukosyl geringer ist als die der Dextrane. Sie wächst in der Reihe Monosaccharid–Disaccharid–Oligosaccharid–Polysaccharid.

Von den einfachen Zuckern haben neben der Isomaltose auch Maltose, Fruktose und Glukose Akzeptor-Wirkung. Die der Saccharose ist so gering, daß sie erst bei hohen Konzentrationen von Einfluß ist.

Diese Ergebnisse wurden in Systemen erzielt, in denen neben den zu untersuchenden Zuckern weitgehend gereinigte Enzympräparate vorlagen. In wachsenden Kulturlösungen entstehen jedoch im Verlauf der Fermentation sowohl hinsichtlich der Art als auch der Menge Zucker mit Akzeptor-Wirkung, die die Synthese unkontrollierbar beeinflussen. Gelenkte Synthesen wurden daher mit Enzymkonzentraten durchgeführt.

Gelenkte Synthese in wachsenden Kulturen

Von Nadel u.a.[5] wurde der Versuch unternommen, eine gelenkte Dextransynthese direkt in der Fermentationslösung vorzunehmen. Neben üblichen Salzen und Eiweiß wurden in der Synthesekultur eine bestimmte Menge Saccharose und eine bestimmte Menge Akzeptor-Dextran vorgelegt und in üblicher Weise beimpft. Es entstand nur etwa 1/3 des gewünschten (klinischen) Dextrans, während die anderen Drittel hochmolekular bzw. niedermolekular waren. Bei der experimentellen Überprüfung der Nadelschen Arbeit und dem Vergleich mit enzymatischen Synthesen erkannten wir, daß Störungen primär von der Impfkultur ausgehen müssen. Wegen des adaptativen Charakters des Enzyms Dextransucrase muß eine Impfkultur als Kohlenstoffquelle Saccharose enthalten. Nach Beimpfen mit *Leuconostoc mesenteroides* treten hier hinsichtlich des Zuckermetabolismus die gleichen Verhältnisse auf wie sie in einer Synthesekultur anzutreffen sind: Es entsteht, da keine substantiellen Mengen Akzeptor-Moleküle vorhanden sind, im wesentlichen hochmolekulares Dextran. Die mit Fortschreiten der Dextransynthese gleichermaßen freiwerdende Fruktose nimmt einen Teil der Glukosyle auf. Es entstehen Leucrose und deren Folgeprodukte. Weiterhin werden durch Glukose Oligosaccharide der Isomaltose-Reihe gebildet. Nach Überimpfen auf eine Synthesekultur, die bestimmte Mengen Saccharose und Akzeptor-Dextran enthält, stören die von der Impfkultur unkontrollierbar eingebrachten Zucker und Dextrane die berechnete Lenkung empfindlich.

In Fig. 1 sind die papierchromatographisch erfaßbaren Zucker wiedergegeben, die entstehen, wenn in einer üblichen Synthesekultur-Lösung neben Saccharose auch andere Zucker mit Akzeptor-Wirkung enthalten sind. Im Falle einer in Gegenwart von Glukose ausgeführten Synthese wurden Isomaltose, Isomaltotriose, Isomaltotetraose, Isomaltopentaose und Leucrose nachgewiesen. In Gegenwart von Fruktose wurden hohe Konzentrationen von Leucrose und in Gegenwart von niedermolekularem Dextran nur geringe Mengen Leucrose gefunden.

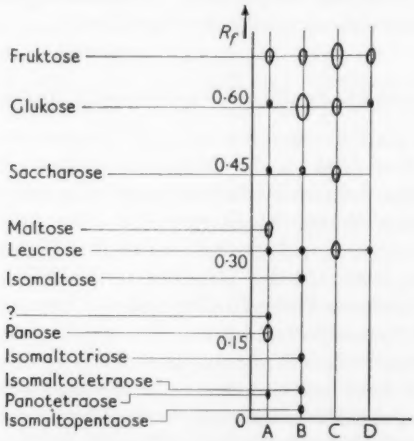

Fig. 1. Die Bildung von Oligasacchariden bei der Dextransynthese unter Zusatz verschiedener Zucker.

Methode. Zu Standard-Nährlösungen* wurden für:

A: 5% Saccharose und 5% Maltose
B: 5% „ „ 5% Glukose
C: 5% „ „ 5% Fruktose
D: 10% „ „ 2% Dextran (*MG* 50,000)

gegeben und im Verhältnis 1:10 mit einer Kultur von *L. mesenteroides* R_1 beimpft. Nach Abfall des pH-Wertes auf 3·9 wurde das Dextran mit Methanol gefällt und die verbliebenen Zucker papierchromatographisch (6) getrennt.

* Standard-Nährlösung:

10% Hefeaufkochung (Hefe : Wasser = 1:5)
0·1% Pepton
0·5% $Na_2HPO_4 \cdot 12\ H_2O$
0·1% KCl

nach Zugabe der entsprechenden Kohlenstoffquelle mit KOH auf pH 7 eingestellt.

Wird mit einer dieser Fermentationslösungen eine Synthesekultur beimpft, die berechnete Mengen Akzeptoren und Saccharosemoleküle enthält, so werden beträchtliche Anteile der Glukosyle durch eingebrachte Akzeptoren aufgenommen.

Der Zusatz von Akzeptor-Dextran, selbst bis zu 4 Prozent, vermag die Ausbeute an klinischem Dextran nicht wesentlich zu steigern. Die Akzeptor-Affinität des mit der Impfkultur einge-

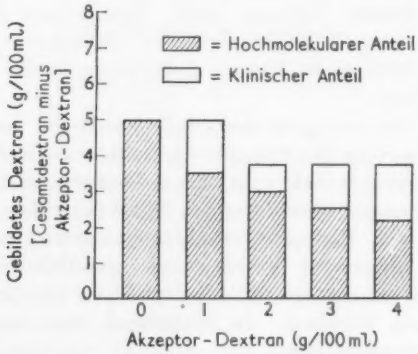

Fig. 2. Abhängigkeit des Verhältnisses klinischen Dextrans zu hochmolekularem Dextran von der Akzeptor-Konzentration bei Beimpfung mit akzeptor- (dextran-) haltiger Impflösung

Methode. Zu Standard-Nährlösungen wurden neben 10% Saccharose steigende Konzentrationen Akzeptor-Dextran (*MG* 50,000) gegeben. Diese Lösungen wurden mit Impfkulturen im Verhältnis 1 : 10 beimpft, in denen nach optimalem Wachstum des *L. mesenteroides* die vorgelegte Saccharose (5%) praktisch in hochmolekulares Dextran und kleinere Anteile niederer Zucker (besonders Leucrose) umgewandelt war. Nach Ende der Synthese wurde eine 1. Fraktion (40% Methanol) als hochmolekularer Anteil und eine 2. Fraktion (52% Methanol) als klinischer Anteil gefällt. Das ausgefällte Dextran wurde gravimetrisch bestimmt. Von den Werten wurde für die graphische Darstellung die eingesetzte Menge Akzeptor-Dextran subtrahiert.

brachten nativen, hochmolekularen Dextrans ist größer als die des Akzeptor-Dextrans, obwohl die Konzentration des von der Impfkultur eingebrachten (nativen) Dextrans in der Synthesekultur nur bei 0·1–0·2 Prozent liegt. Die Lenkung der Synthese kann somit von dem Akzeptor-Dextran nicht wesentlich bestimmt werden.

In Fig. 2 ist der Einfluß des von der Impfkultur eingebrachten

hochmolekularen Dextrans auf die Lenkung zu klinischem Dextran dargestellt. Selbst bei einer relativ hohen Konzentration von 4 Prozent Akzeptor-Dextran blieb der gewünschte klinische Anteil kleiner als 50 Prozent.

Die entscheidende Störquelle ist also das native, hochmolekulare Dextran.

Um in einer wachsenden Kultur eine Lenkung der Synthese zu erreichen, muß die Impflösung frei von Dextran und weitgehend frei von einfachen Zuckern sein. Dies schien anfangs nicht möglich, da wegen des adaptativen Charakters des Enzyms unbedingt substantielle Mengen Saccharose in der Kulturlösung vorliegen sollten.

Es wurde von uns gefunden, daß eine Reihe von organischen Verbindungen vom Nichtzucker-Charakter als Kohlenstoffquelle für dextranbildende Bakterien dienen können und daß selbst nach mehreren Passagen über derartige Nährböden das Vermögen zur Bildung für das Enzymsystem Dextransucrase erhalten bleibt. Es mußten organische Verbindungen gewählt werden, die als Kohlenstoffquelle gut assimilierbar sind und die die Fähigkeit zur Enzymbildung erhalten. In Tabelle I sind einige geeignete Verbindungen aufgeführt.

Tabelle I. Einfluß der Kohlenstoffquelle in der Impfkultur auf die Dextransynthese

Kohlenstoffquelle	Dextran, g/100 ml	Viskosität, η_{rel}
Zitronensäure	3·26	∞
Essigsäure	3·58	
Milchsäure	3·73	
Weinsäure	3·55	
Äthanol	3·78	
Propanol	3·82	
Butanol	3·49	
Saccharose	2·54	72

Methode. Zu Standard-Nährlösungen wurden 0·5 Prozent der oben angeführten Alkohole bzw. Natrium- oder Kaliumsalze der Säuren als Kohlenstoffquelle gegeben und mit dextranfreien Aufschwemmungen von *L. mesenteroides* beimpft. Nach 24-stündiger Inkubation bei 25° wurde die Kultur jeweils einer 10-prozentigen Saccharose-Synthesekultur zugeimpft. Nach Beendigung der Fermentation wurden das Dextran und die Viskosität der Kulturlösung bestimmt. Zum Vergleich wurde mit einer üblichen saccharosehaltigen Impfkultur beimpft.

Impfkulturen, die derartige Kohlenstoffquellen enthalten, sind frei von akzeptorwirksamen Substanzen. Dadurch gelingt eine gelenkte Synthese qualitativ in der gleichen Weise wie mit reinem Enzympräparat. Quantitativ unterscheidet sie sich aber vorteilhaft von einer enzymatischen Synthese dadurch, daß selbst bei hoher Saccharosekonzentration eine fast theoretische Ausbeute erreicht wird.

Die Technische Synthese

Kurze Verfahrensbeschreibung

Die Lenkung der Dextransynthese in einer wachsenden Kultur ermöglicht eine wesentliche Vereinfachung der Technologie. Die Ausbeuten an klinischem Dextran (bezogen auf das im Fermentor gebildete) liegen bei 80 Prozent. Die Fermentationslösung enthält somit fast ausschließlich klinisches Dextran und ist dadurch nur leicht viskos.

Der hohe Ausbeuteindex erlaubt eine ökonomische Herstellung in kleinen Fermentationseinheiten.

Die reproduzierbare Lenkung auf einen vorgewählten Molekulargewichtsbereich erspart die aufwendige Fraktionierung. Kann jedoch, wie im beschriebenen Beispiel, die Dextranlösung nicht opaleszenzfrei filtriert werden, so muß ein möglichst geringer Anteil des Dextrans als 1. Fraktion gefällt werden, damit das Ausfallende die Opaleszenz beseitigt. Dementsprechend muß die Lenkung auf ein etwas höheres Molekulargewicht als das gewünschte erfolgen.

Die günstigen Viskositätsverhältnisse erlauben eine einfache und sehr wirksame Adsorptivbehandlung, die die Lösung von Bakterien und Eiweißen weitgehendst befreit. Zweimaliges Umfällen mit Alkohol (Methanol) beseitigt Reste des Eiweißes und anorganische Salze. Nach der letzten Umfällung wird der Alkohol abgetrieben und die verbleibende, etwa 20-prozentige Dextranlösung sprühgetrocknet.

Die Anlage besteht im wesentlichen im Fermentationsabschnitt aus 4 Jenaer Hängegefäßen von etwa 150 l Inhalt (Fig. 3), im Aufarbeitungsabschnitt aus 2 Fällkesseln von 500 l (Fig. 4) und einem von 1000 l Inhalt sowie einem Sprühtrockner.

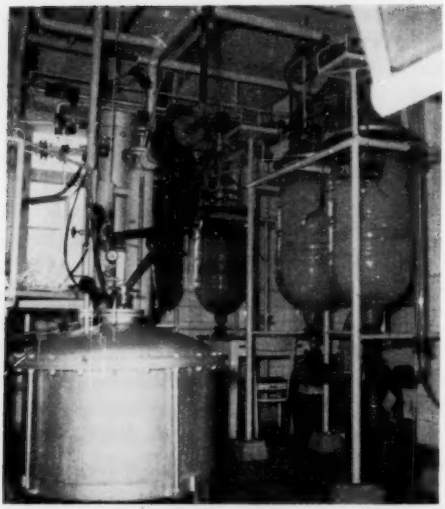

Fig. 3. Fermentationsabschnitt der Dextran-Anlage. Vier 150 l Jenaer Hängegefäße

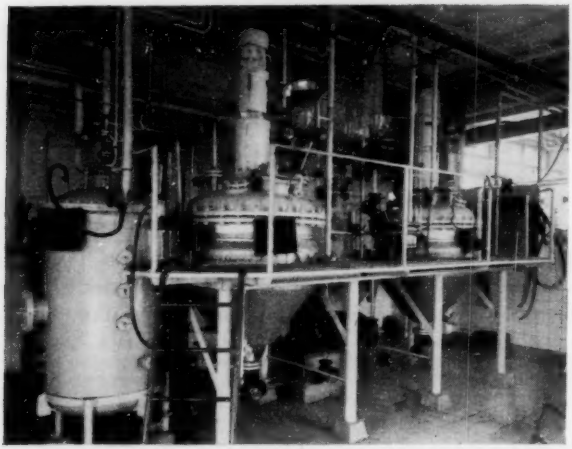

Fig. 4. Aufarbeitungsabschnitt der Dextran-Anlage. Zwei Fällkessel und Zwischenbehälter

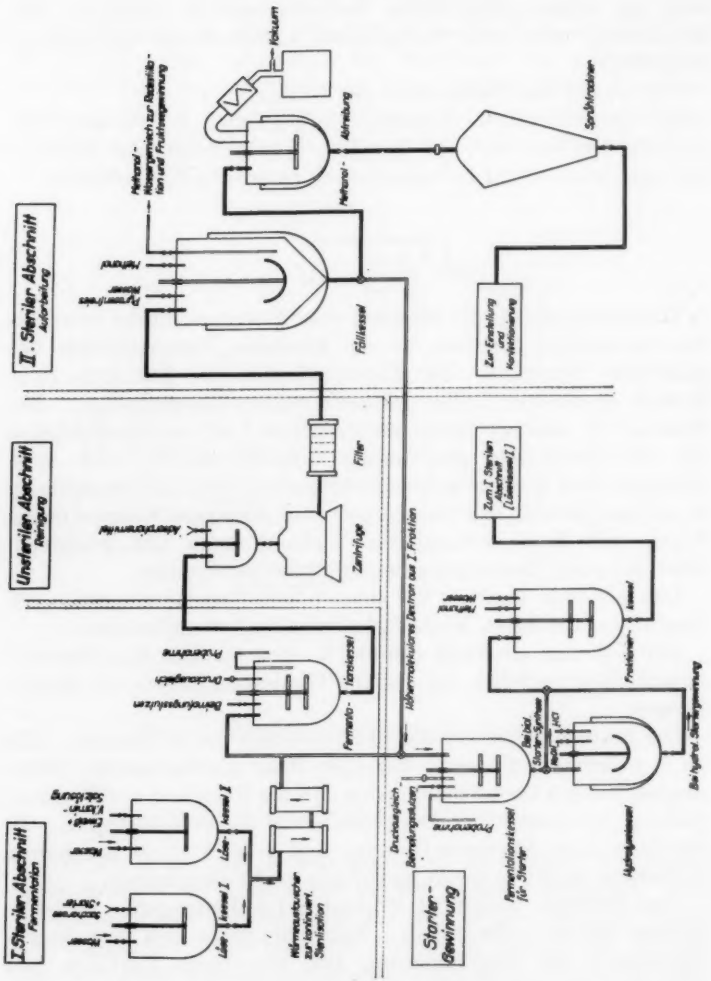

Fig. 5. Fließschema der Dextran-Anlage

Das Akzeptor-Dextran von einem Molekulargewicht um 30,000 wird aus einem gesonderten Betriebsabschnitt bezogen. Die Impflösung wird vom biologischen Laboratorium akzeptorfrei hergestellt.

Fig. 5 zeigt das Fließschema der Anlage.

Bei zweischichtigem Betrieb mit insgesamt 8 Arbeitskräften (16 Stunden/Tag) läßt sich pro Jahr sprühgetrocknetes Dextran für mehr als 60,000 l Infusionslösung (6-prozentig) herstellen.

Stammwahl

Voraussetzung für die Eignung eines Stammes für die Synthese von klinischem Dextran ist die klinische Verträglichkeit des gebildeten Dextrans. Die Besonderheiten der gelenkten Synthese in wachsender Kultur erfordern weitere Eigenschaften. Der Stamm soll niedrige Fermentationszeiten besitzen, die Fähigkeit zur Dextranbildung gleichmäßig aufrechterhalten, auch nach Passagen über saccharosefreie Nährmedien, dem Lenkungsprinzip in weiten Grenzen der Saccharose- und Akzeptor-Konzentration folgen und keine sekundären Veränderungen des gebildeten Dextrans nach Beendigung der Synthese hervorrufen.

Das gebildete Dextran soll einen hohen Prozentsatz von α-1,6-Bindungen aufweisen, leicht löslich und opaleszenzfrei sein.

Der von uns gewählte Stamm *L. mesenteroides* R_1 (Betriebsnomenklatur) erfüllte bis auf die Opaleszenzfreiheit alle Bedingungen.

Die Fermentationszeit des Stammes liegt bei 40 Stunden. Bis zu 8 aufeinanderfolgende Passagen über saccharosefreie Nährmedien werden überstanden, ohne daß die Fähigkeit zur Dextranbildung verlorengeht. Bei Synthesen in Nährlösungen, die 0·5–5 Prozent Akzeptor-Dextran und 5–30 Prozent Saccharose enthalten, wird das Lenkungsprinzip exakt eingehalten.

Das Dextran besitzt 94 Prozent α-1,6-Bindungen. Es ist in kaltem Wasser leicht löslich. Nachteilig wirkt sich eine leichte Opaleszenz der Dextranlösung aus, die durch Umfällen und Adsorptionsfiltration nicht beseitigt werden kann. Durch Fällung eines geringen Prozentsatzes des Gesamtdextrans (5–10 Prozent) läßt sie sich jedoch quantitativ entfernen.

Herstellung der Impflösung

Durch Passagen über akzeptorschwache bzw. akzeptorfreie Nährmedien (Fig. 6) werden die Bakterien von anhaftendem Dextran und die Kulturlösung von Zuckern mit Akzeptor-Wirkung befreit. Die letzte Passage wird in einem üblichen Impfkolben ausgeführt, mit dem dann die Beimpfung der Synthesekultur erfolgt.

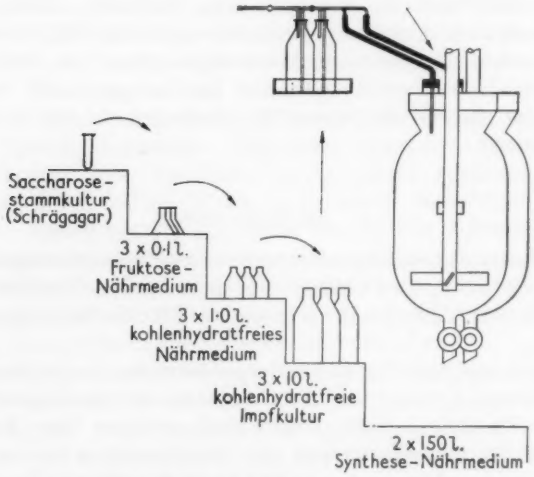

Fig. 6. Schema der Passagen und der Beimpfung

Herstellung von Akzeptor-Dextranen

Dextrane von einem Molekulargewicht von 25,000 bis 30,000 eignen sich gut als Akzeptoren für die gelenkte Synthese. Die Herstellung dieser Starter kann auf drei Wegen erfolgen: Nach dem klassischen hydrolytischen Verfahren lassen sich Nativ-Dextrane durch Säurehydrolyse depolymerisieren. Der wesentliche Nachteil dieser Methode ist die notwendige anschließende Fraktionierung. Es werden zum Fraktionieren unverhältnismäßig große Alkoholmengen (Methanol)—bis auf das 4-fache des

ursprünglichen Volumens—benötigt. Weiterhin setzen sich niedermolekulare Dextrane schlecht ab. Von Lacko und Malek[7] wurde ein Verfahren bekanntgegeben, das darauf beruht, durch Veränderung des pH-Wertes den Alkoholbedarf zu senken.

Nach dem enzymatischen Verfahren läßt sich Dextran durch Pilz- oder Bakterienenzyme (Dextranasen) depolymerisieren.[8, 9] Die Möglichkeit der Gewinnung von Akzeptor-Dextranen auf diesem Wege wird gegenwärtig von uns bearbeitet.

Eine dritte Möglichkeit, die von uns beschrieben wurde,[10] benutzt eine Variation der gelenkten Synthese. Durch Wahl eines besonderen Akzeptors (Glukose) und durch Einhalten einer extrem hohen Saccharose-Konzentration gelingt es, Dextran in einer bimodalen Verteilung derart zu erzeugen, daß der eine Anteil, der größere, mit einem Molekulargewicht bei 20,000 bis 30,000 anfällt.

Synthesemedium

Die Zusammensetzung des Synthesemediums unterscheidet sich nur durch das genau eingestellte quantitative Verhältnis von Saccharose zu Akzeptor-Dextran von dem für die Dextransynthese üblichen.

Die Abhängigkeit des Molekulargewichts des zu synthetisierenden Dextrans (MG_{Dx}) von dem variablen Molekulargewicht des Akzeptor-Dextrans (MG_P), der Konzentration des Akzeptor-Dextrans (c_P in Prozent) und der Konzentration der reaktionsfähigen Glukosyl-Reste (c_{Gl} in Prozent), die sich aus der Saccharose-Konzentration und dem Ausbeuteindex ergibt, ist durch folgende Beziehung gegeben:

$$MG_{Dx} = MG_P\,[(c_{Gl}/c_P +)1]$$

Als Akzeptor ist Dextran von einem Molekulargewicht zwischen 15,000 und 50,000 geeignet, das optimale Molekulargewicht liegt zwischen 25,000 und 30,000. Die Saccharosekonzentration kann bis zu etwa 30 Prozent betragen. Bei höheren Konzentrationen wirkt die Saccharose selbst als Akzeptor; außerdem können osmotische Effekte das Bakterienwachstum ungünstig beeinflussen. Die Saccharose-Konzentration soll möglichst so gewählt werden, daß diese Effekte gerade noch nicht auftreten, um eine möglichst

große Volumenintensität und eine in bezug auf das Akzeptor-Dextran maximale Ausbeute zu erreichen. Bei opaleszierenden Dextranen soll die Lenkung etwas höher erfolgen, statt auf $MG_{Dx} = 75{,}000$ auf $MG_{Dx} = 100{,}000$, damit eine erste Fraktion gefällt werden kann, die opaleszierende Stoffe mitreißt.

Sterilisation

Die entsprechend des mikroaerophilen und acidophilen Verhaltens der dextranbildenden Bakterien nicht belüftete und im sauren pH-Bereich liegende Synthesekultur bietet Endosporen von Bakterien ungünstige Keimungs- und Wachstumsbedingungen. Dementsprechend konnten wir eine vereinfachte Sterilisation wählen, durch die nur die Pilze, die Pilzsporen und die nicht Sporen bildenden Bakterien abgetötet werden. Die Kohlenhydratlösung, die Eiweißlösung und die Salzlösung werden getrennt durch einen Wärmeaustauscher einfachster Bauart geführt. Dieser besteht aus einem Bündel von 7 Rohren von je 1 m Länge und 12 mm Durchmesser, die mit 0·5 atü Betriebsdampf erhitzt werden, so daß beim Durchsatz von 60 l Lösung pro Stunde diese auf 108° gebracht wird. Der Durchsatz beträgt 1 l/min, entsprechend einer Verweilzeit von 45 sec.

Diese relative kurze Sterilisationszeit verhindert das Auskeimen von Sporen für mindestens 50 Stunden. Bakteriologische Überprüfungen ergaben, daß es sich bei den nicht abgetöteten Organismen im wesentlichen um sporenbildende Stäbchen der *Bacillus subtilis*-Gruppe handelt.

Um ein Auswachsen von Fremdorganismen im Wärmeaustauscher, in Leitungen und in den Fermentationskesseln zu vermeiden, werden diese nach jeder Fermentation eine Stunde lang ausgedampft, anschließend 2 Stunden mit formaldehydgesättigter Luft durchgeblasen und danach mit Dampf formaldehydfrei gespült.

Fermentation

Die Fermentation wird bei $23 \pm 2°$ durchgeführt. Der pH-Wert fällt von ursprünglich 6·5–7 auf 3·9. Neben Dextran werden beträchtliche Mengen Fruktose, Leucrose und Mannit gebildet

(Fig. 7). Auffällig ist bei dem hier gewählten Beispiel die wahrscheinlich stammabhängige starke Leucrosebildung, die letztlich auf Kosten des Dextrans vor sich geht.

Die Lenkung der Synthese erfolgt entsprechend den gewählten Konzentrationen an Saccharose und Akzeptor-Dextran. In hunderten von technischen Ansätzen stellte sich das errechnete Molekulargewicht mit nur geringen Abweichungen ein. In

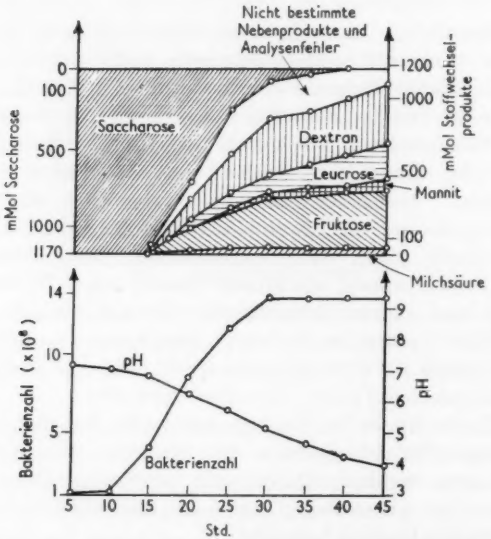

Fig. 7. Biochemische Leistungskurve von *L. mesenteroides* R_1

Methode. Die Bakterienzahl wurde durch Auszählen, die Milchsäure nach Baker und Summerson[11] und Dextran gravimetrisch bestimmt. Die Zucker und Mannit wurden papierchromatographisch getrennt [12, 13] und nach Extrahieren die Zucker nach Roe[14] und Mannit nach Corcoran und Page[15] bestimmt.

Tabelle II sind die erzielten mittleren Molekulargewichte von 21 aufeinanderfolgenden Chargen aus der Dextranproduktion des VEB Serumwerks Bernburg aufgeführt. Die Ausbeute-Indizes in der letzten Spalte führen wir als Beweis dafür an, daß die mittleren Molekulargewichte nicht durch Fraktionierung korrigiert werden mußten.

Tabelle II. Auszug aus dem Produktionsprotokoll der Dextran-Anlage im VEB Serumwerk Bernburg vom September 1959

Charge	Molekulargewicht	Ausbeute (Index)
90	79,000	15
91	65,000	14
92	76,000	17
93	72,000	15
94	74,000	14
95	78,000	15
96	77,000	18
97	76,000	14
98	75,000	15
99	81,000	17
100	77,000	16
101	75,000	17
102	78,000	19
103	81,000	19
104	78,000	18
105	80,000	20
106	75,000	19
107	77,000	19
108	76,000	19
109	63,000	19
110	75,000	19

Die Beziehungen der Molekulargewichtsverteilungen (Fällkurven) zwischen Akzeptor-Dextran, dem Rohdextran in der Kulturlösung und dem klinischen Endprodukt nach den Reinigungs- und Aufarbeitungsschritten sind in Fig. 8 dargestellt. Man erkennt, daß sich die Verteilung des Akzeptor-Dextrans im Prinzip im synthetisierten Rohdextran wiederfindet. Das Rohdextran in der Kulturlösung liegt praktisch bereits im klinischen Bereich. Unerwünschte höhermolekulare Anteile werden nicht gebildet. Der geringe Anteil niedermolekularen Dextrans ist durch die Qualität des Akzeptor-Dextrans bedingt.

Aufarbeitung

Das gebildete Dextran ist als 7 prozentige Lösung nur leicht viskos. Die Reinigung kann daher adsorptiv erfolgen. Zu der Fermentationslösung wird etwa 1 Prozent eines Adsorptionsmittels aus Kieselgur, Zellulose und Asbest gegeben, mit diesem 1 Stunde bei etwa 70°C gut verrührt und in einem Kammerseparator abgeschleudert.

Die Adsorptivbehandlung entfernt die Bakterien und einen Teil des Eiweißes (vgl. Fig. 9). Die Lösung ist jetzt klar, jedoch leicht opaleszent. Die Größe der deswegen zu fällenden 1. Fraktion wird durch Vorversuche bestimmt, so daß mit einem Minimum ausfallenden Dextrans die Opaleszenz beseitigt wird.

Bei Dextranlösungen, die nach Adsorptivbehandlung und Filtration nicht opaleszieren oder deren Verwendungszweck eine Opaleszenz erlaubt, entfällt die Herausnahme einer 1. Fraktion.

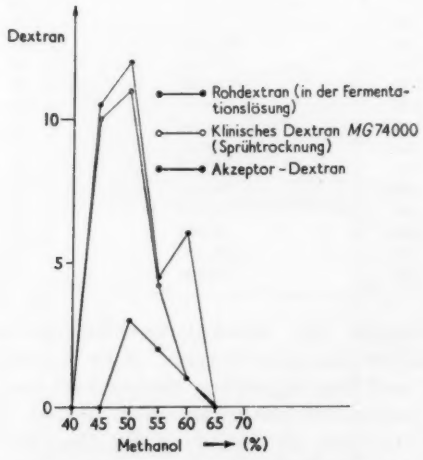

Fig. 8. Molekulargewichtsverteilungen (Fällkurven) von Rohdextran, klinischem Dextran und Akzeptor-Dextran

Methode. 0·5 prozentige Dextranlösungen wurden mit den entsprechenden Mengen Methanol versetzt, auf 40° erwärmt und über 12 Stunden auf 18° abgekühlt. In den überstehenden Lösungen wurde mit Anthron das gelöste Dextran bestimmt und daraus das ausgefallene Dextran errechnet.

Die überstehende Lösung wird von dem Ausgefällten abgehebert und nach Filtration durch Klär- und Sterilschichten in den zweiten Fraktioniertank gedrückt. Hier wird gerade so viel Methanol zugegeben, daß der klinische Anteil ausfällt und von den sich noch in Lösung befindlichen Zuckern, anderen metabolischen Produkten und niedermolekularen Dextranen trennt. Darauf wird noch zweimal umgefällt, um völlige Salzfreiheit zu erreichen (vgl.

Fig. 9). Nach der letzten Umfällung wird die Lösung mit sterilem, apyrogenem Wasser aufgenommen und danach ein Teil des Methanol-Wasser-Gemisches unter Anlegen von Vakuum abdestilliert. Die auf eine Dextrankonzentration von etwa 20 Prozent eingeengte Lösung wird sprühgetrocknet.

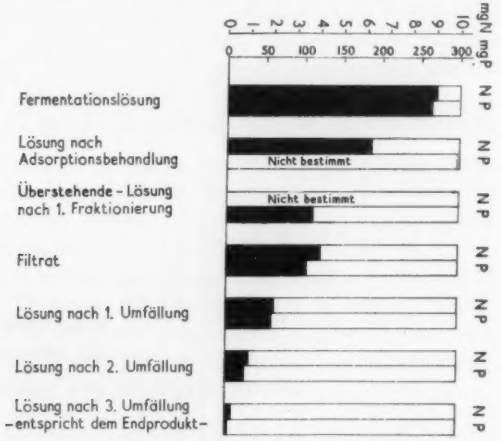

Fig. 9. Abnahme des Eiweiß- und Salzgehaltes in den verschiedenen Aufarbeitungsstufen

Methode. Stickstoff wurde nach Kjeldahl und Phosphat kolorimetrisch nach der Molybdänblau-Methode[16] bestimmt. Die Werte sind auf mg N bzw. mg P in 100 ml 6-prozentiger Dextranlösung berechnet.

Pyrogenität

Die fermentierte Kultur ist in der überwiegenden Zahl der Fälle pyrogen. Durch die Adsorptivbehandlung und die sich daran anschließende Filtration verringert sich die Zahl der pyrogenen Chargen (Fig. 10*a*). Im Verlauf der anschließenden Aufarbeitung nahm die Zahl der pyrogenen Chargen jedoch wieder zu. Bakteriologische Untersuchungen ergaben, daß das für das Auflösen des Dextrans benutzte Wasser zwar pyrogenfrei, jedoch nicht keimfrei war, so daß nach längeren Standzeiten Pyrogene in das Wasser ausgeschieden wurden. Durch Sterilfiltration des Wassers und durch Verringerung der Standzeiten konnte die Pyrogenfreiheit aller Chargen erreicht werden (Fig. 10*b*).

Zusammenfassung

Durch eine spezifisch vorbereitete Impfkultur, die frei von Glukosyl-Akzeptoren ist, gelingt eine Lenkung der Dextransynthese in wachsenden Kulturen.

Die Lenkung erfolgt entsprechend den theoretischen Vorstellungen und kann durch eine einfache Beziehung berechnet werden. Die die Lenkung bestimmenden Faktoren sind Menge und Molekulargewicht des Akzeptor-Dextrans, Ausbeuteindex sowie Menge der Saccharose. Die Molekulargewichtsverteilung des Akzeptor-Dextrans zeichnet sich im gebildeten Dextran wieder ab.

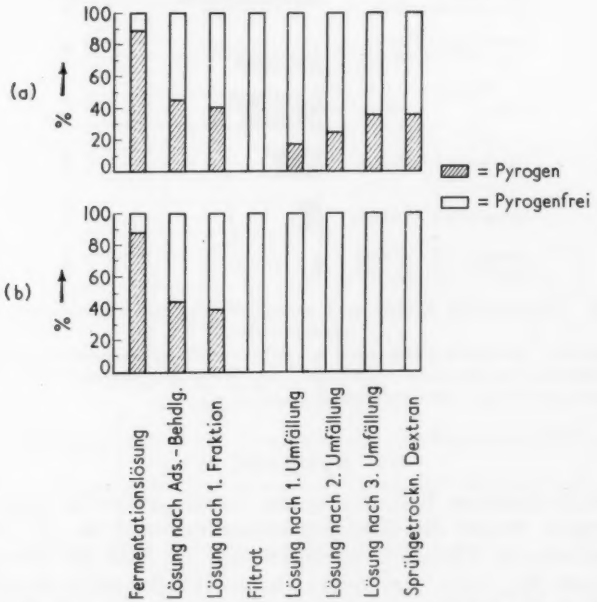

Fig. 10. Veränderung der Pyrogenität in den verschiedenen Aufarbeitungsstufen (*a*) Bei Verwendung von frisch destilliertem, pyrogenfreiem Wasser, (*b*) Bei Verwendung von keimfrei filtriertem, pyrogenfreiem Wasser

Methode. Die Proben aus einer statistischen Zahl von Chargen wurden durch Verdünnen (mit pyrogenfreiem Wasser) auf 6% Dextran eingestellt. Methanolhaltige Proben wurden vorher im Vakuum gereinigt. Die Pyrogenbestimmung wurde nach der *Pharmacopoea internationalis*[17] ausgeführt. Der Prozentsatz der pyrogenen Chargen wurde aufgetragen.

Abhängig von der Molekulargewichtsverteilung des Akzeptor-Dextrans kann das gesamte gebildete Dextran im klinischen Bereich vorliegen. Die Ausbeuten, abhängig von der Saccharosekonzentration, sind höher als sie allgemein bei der Synthese von Nativ-Dextran erhalten werden.

Das Auftreten der für die meisten Dextrane typischen Opaleszenz macht das Ausfällen einer 1. Fraktion notwendig. Das aufwendige Fraktionieren, wie es bei hydrolytischen Verfahren erforderlich ist, entfällt jedoch.

Infolge der günstigen Ausbeuteverhältnisse können volumenmäßig kleine Fermentations- und Aufarbeitungseinheiten benutzt werden, was eine schnelle Anpassung der Produktionskapazität an Marktschwankungen und eine wirtschaftliche Produktion auch geringer Mengen erlaubt. Der volumenmäßige Aufwand beträgt nur 1/5 gegenüber dem hydrolytischen Verfahren.

Neben der Herstellung klinischen Dextrans gewinnt die Herstellung von Dextran mit bestimmtem Molekulargewichtsbereich nach dem Verfahren der gelenkten Synthese in wachsender Kultur zunehmende Bedeutung, insbesondere als Zwischenprodukt für die Herstellung von Dextranderivaten.

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